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(54) Title: DELIVERY OF TREFOIL PEPTIDES

(57) Abstract: The present invention relates to a micro-organism, preferably a bacterial strain, preferably a non-pathogenic strain, preferably a non-invasive strain, preferably a food grade strain, preferably a gram-positive bacterial strain, delivering a trefoil peptide *in vivo*. Preferably said trefoil peptide is TFF1. The present invention further relates to a method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of such a bacterial strain. The present invention also relates to a pharmaceutical composition comprising a trefoil peptide delivering bacterium as well as methods of treatment of acute gastro-intestinal inflammatory diseases comprising administration of said transformed bacterial strains, particularly for treating acute colitis, including but not limited to acute flare-ups of Crohn's disease and ulcerative colitis in humans, as well as for treating gastro-intestinal disorders of a similar nature in other animal species.

### DELIVERY OF TREFOIL PEPTIDES

The present invention relates to the field of *in vivo* protein delivery systems. More particularly, the present invention relates to the secretion *in vivo* of trefoil peptides by micro-organisms, preferably bacterial strains, preferably non-pathogenic strains, preferably non-invasive strains, preferably food grade strains, methods for delivering trefoil peptides using said systems and the use of said trefoil peptide expression systems for treatment of inflammatory disorders of the gastro-intestinal tract.

*Lactococcus lactis* is a Gram-positive non-pathogenic lactic acid bacterium which can survive in the intestine (Klijn *et al.*, 1995). It is not certain whether *L. lactis* can also be metabolically active in all of these environments.

The expression of tetanus toxin fragment C by *Lactococcus lactis* in view of vaccination was described by Wells *et al.* (1993b) and Robinson *et al.* (1997). Further, it was demonstrated that when preparations of *L. lactis* bacteria engineered to express either Interleukin-2 or Interleukin-6 together with tetanus toxin fragment C (TTFC) were administered intranasally to mice, more than 10 times more anti-TTFC was produced than after similar administration of strains expressing TTFC alone (International patent application published under WO 97/14806). These results prove the use of a cytokine-secreting, non-invasive experimental bacterial vaccine vector to enhance immune responses to a co-expressed antigen. Also an approach has been described to attach heterologous protein fragments in the cell wall and by this way display them at the *L. lactis* surface, possibly leading to more enhanced vaccination properties (WO 97 09437 Steidler, Remaut, Wells).

Trefoil peptides are secreted by epithelial mucus cells and are stable in an acid environment. These peptides contribute to the protection of the mucosa (formation of a gel over the epithelium) and are probably involved in the repair of damaged mucosa by stimulation of epithelial migration (Playford *et al.*, 1996). The production of trefoil peptides increases locally in regions where damage occurs such as gastric ulcers and colitis (Wright *et al.*, 1990). Babyatsky *et al.* (1996) have shown that the administration of recombinant trefoil peptides reduces the damage at those places. In contradiction with most other proteins that are important for the protection of the mucosa (such as epidermal growth factor), most studies have demonstrated that trefoil peptides cause little or no proliferation (Playford *et al.*, 1996). Three members of this family of trefoil peptides have been identified in humans and originally designated: pS2 (breast cancer oestrogen inducible gene, O. Lefebvre, 1993), SP (spasmolytic peptide) and ITF

(intestinal trefoil factor). In the present nomenclature pS2 is renamed as TFF1, SP as TFF2 and ITF as TFF3 (see e.g. Wong *et al.*, 1999). This new nomenclature will be used throughout the present text.

In humans, mice and rat TFF1 and TFF2 are predominantly found in the stomach while TFF3 is predominantly found in the duodenum and colon. Wong *et al.* (1999) give a recent overview of trefoil peptides. The contents of this article are incorporated by reference in the present disclosure.

TFF1 is thought to act through a cell surface receptor (Tan *et al.*, 1997).

The use of trefoil proteins or peptides for treatment of disorders of and damage to the alimentary canal, including the mouth, oesophagus, stomach, and large and small intestine, as well as for the protection and treatment of tissues that lie outside the alimentary canal are described in WO 97/38712 and WO 92/14837. These proteins can be used either to treat lesions in these areas or to inhibit the formation of lesions. These lesions can be caused by: radiation therapy or chemotherapy for the treatment of cancer, any other drug including alcohol which damages the alimentary canal, accidental exposure to radiation or to a caustic substance, infection, a digestive disorder including but not limited to non-ulcer dyspepsia, gastritis, peptic or duodenal ulcer, gastric cancer, MALT lymphoma, Menetier's syndrome, gastro-oesophageal reflux disease, Crohn's disease, ulcerative colitis and acute colitis of chemical, bacterial or obscure origin.

Trefoil peptides are particularly useful to treat acute colitis.

ITF has also been used in combination with EGF (epidermal growth factor) for treating gastro-intestinal tract ulcers. *In vitro* and *in vivo* experiments have shown that the wound healing activities of EGF are markedly increased by treatment of EGF in combination with ITF, without increasing the proliferative action of EGF (Chinery and Playford, 1995).

Inflammatory bowel disease is the group name for a range of gastro-intestinal inflammations. Belonging to this group are enteritis, colitis, inflammations of respectively the mucosa of the duodenum or the colon. Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa) are closely related, chronic and spontaneously recurring diseases of the gastro-intestinal tract. These diseases are immunologically mediated and have environmental and genetic causes. Sartor (1995) describes the different aspects of inflammatory bowel disease. Crohn's disease has been particularly studied by for instance Herfath and Sartor, (1994), Cominelli *et al.* (1994), and MacDermott (1989).

The aim of the present invention is to provide a method for delivering trefoil peptides to treat gastro-intestinal disorders.

Another aim of the present invention is to provide a pharmaceutical composition for treating gastro-intestinal disorders.

- 5       The present invention relates more particularly to a micro-organism delivering a trefoil peptide *in vivo*. Preferentially said micro-organism is a bacterial strain, preferably a non-pathogenic strain, preferably a non-invasive strain, preferably a food grade strain, more preferably a gram-positive bacterial strain, most preferably a lactic acid fermenting bacterial strain, preferably a *Lactococcus* or a *Lactobacillus* species
- 10       expressing a trefoil peptide *in vivo*. The present invention is thus applicable to any of the *Lactococcus* or *Lactobacillus* species or subspecies selected from the list comprising *Lactococcus garvieae*, *Lactococcus lactis*, *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *hordniae*, *Lactococcus lactis*, *Lactococcus lactis* subsp. *Lactis*, *Lactococcus piscium*, *Lactococcus plantarum*, *Lactococcus raffinolactis*,
- 15       *Lactobacillus acetotolerans*, *Lactobacillus acidophilus*, *Lactobacillus agilis*, *Lactobacillus algidus*, *Lactobacillus alimentarius*, *Lactobacillus amyolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylovorus*, *Lactobacillus animalis*, *Lactobacillus aviarius*, *Lactobacillus aviarius* subsp. *araffinosus*, *Lactobacillus aviarius* subsp. *aviarius*, *Lactobacillus bavaricus*, *Lactobacillus bifementans*, *Lactobacillus brevis*,
- 20       *Lactobacillus buchheri*, *Lactobacillus bulgaricus*, *Lactobacillus carnis*, *Lactobacillus casei*, *Lactobacillus casei* subsp. *alactosus*, *Lactobacillus casei* subsp. *casei*, *Lactobacillus casei* subsp. *pseudopiantarum*, *Lactobacillus casei* subsp. *rhamnosus*, *Lactobacillus casei* subsp. *tolerans*, *Lactobacillus cateniformis*, *Lactobacillus cellobiosus*, *Lactobacillus collinoides*, *Lactobacillus confusus*,
- 25       *Lactobacillus coryniformis*, *Lactobacillus coryniformis* subsp. *coryniformis*, *Lactobacillus coryniformis* subsp. *torquens*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus curvatus* subsp. *curvatus*, *Lactobacillus curvatus* subsp. *melibiosus*, *Lactobacillus delbrueckii*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus delbrueckii* subsp. *lactis*,
- 30       *Lactobacillus divergens*, *Lactobacillus farciminis*, *Lactobacillus fermentum*, *Lactobacillus fornicalis*, *Lactobacillus fructivorans*, *Lactobacillus fructosus*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus graminis*, *Lactobacillus halotolerans*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus heterohiochii*, *Lactobacillus hilgardii*, *Lactobacillus homohiochii*, *Lactobacillus iners*,
- 35       *Lactobacillus intestinalis*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kandleri*, *Lactobacillus kefir*, *Lactobacillus kefiranoferiens*, *Lactobacillus kefirgranum*,

*Lactobacillus kunkeei*, *Lactobacillus lactis*, *Lactobacillus leichmannii*, *Lactobacillus lindneri*, *Lactobacillus malefermentans*, *Lactobacillus mali*, *Lactobacillus maltaromicus*, *Lactobacillus manihotivorans*, *Lactobacillus minor*, *Lactobacillus minutus*, *Lactobacillus mucosae*, *Lactobacillus murinus*, *Lactobacillus nagellii*, *Lactobacillus oris*, *Lactobacillus*  
 5 *panis*, *Lactobacillus parabuchneri*, *Lactobacillus paracasei*, *Lactobacillus paracasei*  
 subsp. *paracasei*, *Lactobacillus paracasei* subsp. *tolerans*, *Lactobacillus parakefiri*,  
*Lactobacillus paralimentarius*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus*,  
*Lactobacillus perolens*, *Lactobacillus piscicola*, *Lactobacillus plantarum*, *Lactobacillus*  
 10 *pontis*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Lactobacillus rimae*,  
*Lactobacillus rogosae*, *Lactobacillus ruminis*, *Lactobacillus sakei*, *Lactobacillus sakei*  
 subsp. *carneus*, *Lactobacillus sakei* subsp. *sakei*, *Lactobacillus salivarius*,  
*Lactobacillus salivarius* subsp. *salicinii*, *Lactobacillus salivarius* subsp. *salivarius*,  
*Lactobacillus sanfranciscensis*, *Lactobacillus sharpeae*, *Lactobacillus suebicus*,  
*Lactobacillus trichodes*, *Lactobacillus uli*, *Lactobacillus vaccinostrercus*, *Lactobacillus*  
 15 *vaginalis*, *Lactobacillus viridescens*, *Lactobacillus vitulinus*, *Lactobacillus xylosus*,  
*Lactobacillus yamanashiensis*, *Lactobacillus yamanashiensis* subsp. *mali*,  
*Lactobacillus yamanashiensis* subsp. *Yamanashiensis* and *Lactobacillus zeae*.

It was not obvious from the capacity of *Lactococcus lactis* to deliver one  
 heterologous antigen or its ability to produce molecules such as IL-2 and IL-6 *in vitro*  
 20 and *in vivo* that bacteria would be an appropriate vehicle for delivery of other types of  
 peptides or polypeptides *in vivo*. Further it is unknown whether said trefoil peptides  
 expressed by said bacterial strains will provide a beneficial effect to inflammatory  
 diseases of the gastro-intestinal tract, such as inflammatory bowel disease or acute  
 colitis.

It is, therefore, surprising that it could be demonstrated in the present Examples  
 section that bacterial strains are able to express trefoil peptides *in vivo* when present in  
 the gastro-intestinal canal and exert a healing effect in acute colitis situations. By way  
 of example, PCR fragments containing the coding region mouse TFF1 were cloned.  
 Recombinant vectors comprising these PCR clones under the control of a promotor  
 30 and the *usp45* *Lactococcus lactis* secretion signal sequence were constructed.  
 Transformed *Lactococcus lactis* strains were constructed which express mouse TFF1  
 trefoil peptides. It was further shown in an *in vivo* mice model system that recombinant  
 mTFF1 produced by these bacteria can surprisingly exert healing effects on the distal  
 part of the inflamed colon.

35 According to a preferred embodiment, the present invention relates particularly  
 to a bacterial strain delivering trefoil peptide *in vivo*.

According to another preferred embodiment, the present invention relates to a bacterium delivering TFF1 *in vivo*.

It is to be understood that the present invention also relates to parts or variants of any trefoil peptide. Said parts refer to biologically active parts which can be generated by methods known to those skilled in the art. These parts will generally contain at least 10 contiguous amino acids, typically at least 20 contiguous amino acids, more typically at least 30 contiguous amino acids, usually at least 40 contiguous amino acids, and preferably at least 50 contiguous amino acids. Said variants refer to variants which have the same biological activity as the above mentioned trefoil peptides.

It should also be clear that bacterial strains according to the present invention as defined above, may also express additional recombinant proteins which are beneficial to the treatment of any envisaged disorder.

According to yet another embodiment, the present invention relates to a pharmaceutical composition comprising a micro-organism expressing a trefoil peptide as defined above.

Advantageously, the pharmaceutical composition according to the present invention is preferably suitable for application to mucosal surfaces.

Pharmaceutical compositions according to the present invention, and for use in accordance to the present invention, may comprise, in addition to the micro-organism, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration. Those of relevant skill in the art are well able to prepare suitable solutions.

According to another embodiment, the present invention relates to a method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of a micro-organism as defined above.

According to another aspect, the present invention also relates to the use of a micro-organism as defined above for the manufacture of an agent for the delivery of trefoil peptide to the gastro-intestinal tract.

According to another embodiment, the present invention relates to a method of treatment of gastric and/or intestinal diseases and/or disorders comprising administration of a micro-organism as defined above.

The present invention also relates to a method of treatment of gastric and/or intestinal diseases and/or disorders comprising administration of a micro-organism delivering a TFF1 trefoil peptide *in vivo*.

5 The trefoil proteins expressed by the bacterial strains according to the present invention can be used either to treat lesions in these areas or to inhibit the formation of lesions caused by gastro-intestinal diseases and disorders.

The expression "gastric and/or intestinal diseases and/or disorders" relates to all types of gastric, intestinal and gastro-intestinal diseases and/or disorders. In preferred embodiments of the invention this expression relates to acute gastro-intestinal inflammatory diseases and disorders. These diseases are preferably acute  
10 gastro-intestinal disorders of chemical, bacterial or obscure origin. Belonging to this group are enteritis, colitis, including but not limited to acute flare-ups in Crohn's disease and ulcerative colitis inflammations of, respectively, the mucosa of the duodenum or the colon. Also included herewith is traveller's disease. In other preferred  
15 embodiments of the invention the expression "gastric and/or intestinal diseases and/or disorders" relates to chronic and spontaneously recurring diseases of the gastro-intestinal tract such as Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa).

The expression "gastric and/or intestinal diseases and/or disorders" also relates  
20 to diseases involving lesions at mucosal surfaces. As such, the disease states to be treated by the methods and pharmaceutical compositions of the invention can also include disorders of and damage to the alimentary canal, including the mouth, oesophagus, stomach, and large and small intestine, as well as for the protection and treatment of tissues that lie outside the alimentary canal. These lesions can be caused  
25 by: radiation therapy or chemotherapy for the treatment of cancer, any other drug including alcohol which damages the alimentary canal, accidental exposure to radiation or to a caustic substance, infection, a digestive disorder including but not limited to non-ulcer dyspepsia, gastritis, peptic or duodenal ulcer, gastric cancer, MALT lymphoma, Menetier's syndrome, gastro-oesophageal reflux disease, and Crohn's  
30 disease.

The present invention thus relates to the use of a micro-organism as described above for the preparation of a medicament for treatment of gastric and/or intestinal diseases and/or disorders.

The present invention also relates to the use of a micro-organism as described  
35 above for the preparation of a medicament for treatment of acute gastro-intestinal inflammatory diseases, acute colitis, acute flare-ups of Crohn's diseases and ulcerative

colitis, and for treatment of chronic and spontaneously recurring diseases of the gastro-intestinal tract comprising Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa).

According to another embodiment, the invention relates to the use of a micro-  
5 organism as described above for the preparation of a medicament for inhibiting the formation of lesions caused by gastric and/or intestinal diseases and disorders.

Administration of the micro-organism may be orally or by means of any other method known in the art allowing the micro-organism to enter the desired places to be treated, such as e.g. anal, vaginal. The micro-organism may be applied in a nutrient  
10 medium, i.e. a medium containing a substance or substances which sustain (at least *in vitro*) metabolic activity of the micro-organism. Such substances may sustain viability if not growth of the micro-organism. Such substances may include an energy source such as glucose, amino acids and so on.

The individual to which the micro-organism is administrated may be a human or  
15 an animal.

In a therapeutic context, i.e. where the biological effect of delivery of the polypeptide to an individual is beneficial to that individual, administration is preferably in a 'therapeutically effective amount', this being sufficient to show benefit to the patient. Such benefit may be at least amelioration of one symptom. The actual amount  
20 administered, and rate and time-course of administration, will depend on the aim of the administration, e.g. the biological effect sought in view of the nature and severity of the challenge and is the subject of routine optimisation. Prescriptions of treatment, for example decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

25 A composition comprising micro-organisms according to the present invention may be administered in accordance with the present invention alone or in combination with other treatments, either simultaneously or sequentially.

According to another embodiment, the present invention relates to a method for producing a micro-organism delivering a trefoil peptide *in vivo* as defined above  
30 comprising transforming a micro-organism with a recombinant vector carrying a trefoil polypeptide coding sequence under the control of a suitable promoter and a suitable bacterial secretion signal sequence.

Said bacterial secretion signal sequence can be any sequence known in the art to perform said function. Preferably, for *L. lactis* said secretion signal is the *usp45* *L. lactis* secretion signal sequence. Said promoter sequence can be any promoter  
35 allowing expression of said coding sequence in said micro-organism. Examples given



in the examples section include the known inducible *E. coli* phage T7 promoter and the known constitutive P1 promoter of *L. lactis*.

5 The present invention also relates to a recombinant vector comprising at least a part of a trefoil peptide coding sequence under the control of a suitable promoter and a suitable secretion signal sequence. Said recombinant vector can be used to deliver *in vivo* at least a part of a trefoil peptide sequence which can exert on healing effect on damaged areas of the mucosal surfaces.

The present invention further relates to a recombinant vector as defined above, having a nucleotide sequence as represented by any of SEQ ID NOs 1, 2 or 4.

10 The following examples merely serve to illustrate the present invention, and are not to be construed as limiting the invention in any way.

All documents mentioned in this text are incorporated by reference.

## FIGURE LEGENDS

**Figure 1:** Overview of the plasmids used.

**Figure 1a :** Schematic maps of the plasmids pL2mTFF1v1, and pT1mTFF1. T7 is the major late promoter from coliphage T7 (Studier and Moffatt, 1986). P1 is the lactococcal promoter as in Waterfield *et al.*, (1995), usp45S is a DNA fragment encoding the secretion signal peptide from the lactococcal Usp45 protein (van Asseldonck *et al.*, 1990), mtff1 is a DNA fragment encoding the mature part of murine TFF1, mtff1v1 is a DNA fragment encoding a truncated (missing two aminoterminal aa residues) mature murine TFF1, Cm is the chloramphenicol selection marker, Em is the erythromycin selection marker. For pPICmTFF1 : PPMF is the prepro *Saccharomyces cerevisiae*  $\alpha$ -mating factor; AOX1 prom is the alcohol oxidase promotor; AOX1 term is the alcohol oxidase terminator; HIS4 is the Histidol dehydrogenase gene; Ori is an *Escherichia coli* origin of replication; AOXfr is a 3' fragment of the alcohol oxidase gene; AmpR is the ampicillin resistance gene, All components are from the pPIC9 plasmid (Invitrogen).

**Figure 1b :** DNA sequence of plasmid pL2mTFF1v1 (SEQ ID NO 1).

**Figure 1c :** DNA sequence of plasmid pT1mTFF1 (SEQ ID NO 2).

**Figure 1d :** DNA sequence of plasmid pPICmTFF1 (SEQ ID NO 3)

**Figure 2:** SDS-PAGE. The different protein fractions are derived from the medium of *L. lactis* MG1820 [pILPOL] (control), MG1820 [pILPOL; pL2mTFF1v1] , MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells. The two left lanes contain marker proteins wherein the molecular weight is given in kDa. The proteins were visualised using Coomassie Blue staining.

**Figure 3:** Representation of the histological scores of the distal part of the colon. Top left hand side graphic: epithelium damage (distal part colon). Top right hand side graphic: inflammatory infiltration (distal part colon). Bottom graphic: sum of the histological scores of the top graphics (distal part colon).

- Figure 4 :** Representation of the histological scores of the distal part of the colon of healthy mice (control) or mice with acute DSS colitis without treatment (DSS) or after treatment with MG1363, MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells.
- 5 **Figure 5 :** Pro-inflammatory cytokine titrations in acute inflamed colon tissue. Interleukin-1 $\beta$  in distal colon (left) and interferon- $\gamma$  in middle and distal colon (right) of healthy mice (control) or mice with acute DSS colitis without treatment (DSS) or after treatment with MG1363, MG1363 [pTREX1] or MG1363 [pT1mTFF1] cells.
- 10 **Figure 6 :** SDS-PAGE of protein fractions from the medium of selected *Pichia pastoris* (GST115::pPICmTFF1) and negative control. The mTFF1 producer clone which was further used for production of mTFF1 is indicated by an arrowhead. The proteins were visualised using Coomassie Blue staining.
- 15 **Figure 7 :** A: Gelfiltration pattern of purified mTFF1 (Superdex 75; Pharmacia). The mTFF1 protein eluted in two peaks with the majority being present in fractions 14, 15, 16 (dimer) and 20 (monomer). The identity of the protein in these fractions was shown to be mTFF1 by SDS-PAGE (insert). The proteins were visualised using Coomassie Blue staining. B: reducing and
- 20 non-reducing SDS-PAGE of purified mTFF1. Left lanes are size markers of indicated sizes, Coomassie Brilliant Blue staining.
- Figure 8:** Representation of the histological scores of the distal part of the colon of mice treated by intraperitoneal injection (i.p.), oral (oral) and rectal (rectal) inoculation, before (pre), during (du) or after (po) installation of acute
- 25 DSS-induced colitis. DSSdu represents scores of PBS treated mice induced for acute DSS colitis.

## EXAMPLES

### Example 1: Cloning and expression of mouse TTF1 (mTTF1)

#### 5 **Culture media**

GM17 is M17 (Difco, Detroit) supplemented with 0.5 w/v % of glucose. M9 medium contains per litre: 6g of Na<sub>2</sub>HPO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 1 g of NH<sub>4</sub>Cl, 0.5 g of NaCl, 2 mmol of MgSO<sub>4</sub>, 0.1 mmol of CaCl<sub>2</sub> and 5 g of Casitone (Difco). M9B is M9 supplemented with 2.1 g of NaHCO<sub>3</sub> and 2.65 g of Na<sub>2</sub>CO<sub>3</sub> per liter. GM9B is M9B supplemented with 0.5 w/v % of glucose. LM9B is M9B supplemented with 0.5 w/v % of lactose.

When appropriate the antibiotics, erythromycin (Er) or chloramphenicol (Cm), were added to the respective media at final concentrations of 5 µg/ml each. The designation used to indicate the presence of antibiotic is, e.g. GM17Er, LM9BCm and so on. Solid media contained 1.2 % agar.

#### **Recombinant DNA techniques**

DNA modifying enzymes and restriction endonucleases were used under standard conditions and in the buffers recommended by the manufacturers. General molecular cloning techniques and the electrophoresis of DNA and proteins were carried out according to standard procedures. *L. lactis* was transformed by electroporation of cells grown in the presence of glycine (Wells *et al.*, 1993a). Plasmid DNA was routinely purified using the Qiagen Plasmid Kit

#### 25 **PCR amplification of mTTF1**

The PCR reaction was carried out on a plasmid containing mTTF1 cDNA (Lefebvre, 1993) using the oligonucleotide primers mTTF1S and mTTF1A. The mTTF1S primer corresponds to the first 18 nucleotides of the sense strand of *mTTF1* from the first nucleotide behind the signal sequence. The mTTF1A primer is complementary to the last 26 nucleotides of the sense strand of *mTTF1* including the stop codon, and introduces an extra *SpeI* restriction site.

mTTF1S: 5'-CAGGCCAGCCAGGCC-3' (SEQ ID NO 4)

mTTF1A: 5'-GCACTAGTTAGAAGGGACATTCTTCTTG AG-3' (SEQ ID NO 5) wherein ACTAGT in mTTF1A represents an *SpeI* site

PCR amplification was carried out using Vent™ DNA polymerase (New England Biolabs (Beverly, USA) which gives a PCR product carrying blunt ends. The PCR mixture consisted of 2 units Vent DNA polymerase, 10µl Vent buffer (thermopol), 4µl dXTP's (0.5mM maximum), 5µl (0.5µM) of each primer, 1µl (50 ng) template DNA  
5 and 74µl H<sub>2</sub>O. Six reactions were set up differing in their final concentration of MgSO<sub>4</sub>, adjusted to 0, 1, 2, 3, 4 and 5 mM respectively. PCR amplification cycles were: T<sub>0</sub> for 300" at 94°C, T<sub>1</sub> for 45" at 94°C, T<sub>2</sub> for 30" at 60°C, T<sub>3</sub> for 20" at 72°C, T<sub>4</sub> for 10" at 20°C. These cycles T<sub>1</sub> until T<sub>3</sub> were carried out 30 times.

PCR amplification with these primers rendered the gene for mature *mTFF1* lacking  
10 the signal sequence and including an additional *SpeI* restriction site. After checking by gel electrophoresis, the amplified fragment appeared as a band in the expected length range. The 5' end of the *mTFF1* sequence contains two possible target sequences complementary to the forward primer. As a consequence two fragments of 202 base pairs and 208 base pairs respectively can be amplified from the *mTFF1* cDNA by use  
15 of the mentioned primers. These fragments are not expected to be resolved by agarose gel electrophoresis.

### **Construction of plasmids**

Two different types of vectors were used as acceptors for the *mTFF1* trefoil  
20 peptide encoding PCR fragment. The primary structure of the two parental vectors - pT1NX, derived from pTREX1 (Wells and Schofield, 1996), and pLET2NX, derived from pLET2N (Steidler *et al.*, 1995) - contains the following common elements: a promoter (T7 or P1), the *L.lactis usp45* secretion signal sequence (van Asseldonk *et al.*, 1990 and European patent application published under No. 0 455 280), modified to  
25 contain a *NaeI* restriction site overlapping the sequence encoding the ultimate aa residue (Steidler *et al.*, 1995), and a downstream *SpeI* restriction site. pT1NX derived plasmids specify resistance to erythromycin; pLET2NX derived plasmids specify resistance to chloramphenicol. The PCR fragments were treated for 1 hour at 37°C using 50µl DNA solution, 10µl *SpeI*-buffer, 50 units *SpeI*, 10 units T4 polynucleotide  
30 kinase (Gibco BRL, Bethesda, USA), 0.5 mM ATP, adjusted to pH 7.5, and 36µl H<sub>2</sub>O. The vector pT1NX was digested for 1 hour at 37°C using 10 à 20µl purified DNA, 10µl *NaeI* buffer, 10 units *NaeI*, 50 units *SpeI*, 1 unit calf intestine alkaline phosphatase (Boehringer, Mannheim, Germany) and 73 à 63µl H<sub>2</sub>O. After 30 minutes incubation, 50 units of *SpeI* and 10 units of *NaeI* were again added to the mixture. The restriction  
35 enzymes were inactivated and extracted from the mixture by phenol/chloroform extraction. After restriction digestion, the *mTFF1*-derived band (comprising a 195 bp

and a 201 bp fragment as described before under "PCR amplification of mouse TFF1 (mTFF1)", and the vector parts were excised from the agarose gel. Following ligation of the respective PCR fragments and the vector for 45 minutes at 16°C using "Ready To Go" T4 DNA ligase (Pharmacia Biotech, UK) recombinant plasmids were obtained  
5 containing the mTFF1 cistron as an in-frame fusion to the *usp45* secretion signal sequence under the control of the promoter.

The plasmid pT1mTFF1 (Figure 1a), which contains the constitutive *L. lactis* P1 promoter, resulted from ligation of the purified *NaeI* - *SpeI* vector part of pT1NX and the *SpeI* cut and 5' phosphorylated PCR fragment.

10 The plasmid pL2mTFF1v1 (Figure1a), which contains the inducible *E. coli* phage T7 promoter, resulted from ligation of the purified *NaeI* - *SpeI* vector part of pLET2N and the *SpeI* cut and 5' phosphorylated PCR fragment. The T7 promoter can only be activated by the cognate T7 RNA polymerase encoded by e.g. plasmid pILPOL. This plasmid is present in *L. lactis* strain MG1820 [pILPOL] (Wells *et al.*,  
15 1993c).

For structural analysis plasmid pT1mTFF1 was transformed into *L. lactis* strain MG1363. The cells were grown on GM17Er plates. Colonies were grown in 2.5 ml GM17Er and the plasmid was isolated. By means of an analytical digest, the restriction pattern of the pT1NX vector (2µl DNA (pT1NX), 20 units *EcoRI*, 50 units *SpeI*, 2µl  
20 *SpeI*-buffer and 15µl H<sub>2</sub>O) and the isolated recombinant plasmid (5µl DNA, 20 units *EcoRI*, 50 units *SpeI*, 2µl *SpeI*-buffer, 0.25 µl of a 10 µg/ml Rnase A stock solution, 12µl H<sub>2</sub>O) were compared. The plasmids were cut with *EcoRI* and *SpeI* for 1h at 37°C. In the reference plasmids, two linear fragments of 907bp and 4999bp are predicted. In pT1mTFF1, two bands of 499 bp and 4999 bp are predicted. The sizes of the  
25 experimentally obtained fragments, as visualized by agarose gel electrophoresis and EtBr staining, were consistent with the predicted lengths. From each recombinant plasmid, one positive culture was streaked out on GM17Er plates to obtain isolated colonies. One colony was subsequently inoculated in 100 ml GM17Er medium and grown to saturation. The cells were collected and the plasmids were purified. Their  
30 physical structure was verified by restriction enzyme analysis and agarose gel electrophoresis. In addition, sequence analysis revealed that the *mTFF1* cistron had been ligated perfectly in frame with the *usp45* secretion leader sequence. pT1mTFF1 contains a 208 bp insert which represents the complete coding sequence of mature mTFF1 (as described before under "PCR amplification of mouse TFF1 (mTFF1)").

35 For structural analysis plasmids pL2mTFF1v1 was transformed into strain MG1820[pILPOL]. The cells were grown on GM17Cm plates. Colonies were grown in

2.5 ml GM17Cm and the plasmids were isolated. By means of an analytical digest, the restriction pattern of the pLET2NX vector (2µl DNA (pLET2NX), 20 units *EcoRI*, 50 units *SpeI*, 2µl *SpeI*-buffer and 15µl H<sub>2</sub>O) and the isolated recombinant plasmid (5µl DNA, 20 units *EcoRI*, 50 units *SpeI*, 2µl *SpeI*-buffer, 0.25 µl of a 10 µg/ml Rnase A stock solution, 12µl H<sub>2</sub>O) were compared. The recombinant plasmid was cut with *EcoRI* and *SpeI* for 1h at 37°C. In the reference plasmids, two linear fragments of 907bp and 4650bp are predicted. In pL2mTFF1, two bands of 499 bp and 4650 bp are predicted. The sizes of the experimentally obtained fragments, as visualized by agarose gel electrophoresis and EtBr staining, were consistent with the predicted lengths. From the recombinant plasmid, one positive culture was streaked out on GM17Cm plates to obtain isolated colonies. One colony was subsequently inoculated in 100 ml GM17Cm medium and grown to saturation. The cells were collected and the plasmid was purified. Its physical structure was verified by restriction enzyme analysis and agarose gel electrophoresis. In addition, sequence analysis revealed that the *mTFF1* cistron had been ligated in frame with the *usp45* secretion leader sequence. The analysis further showed that pL2mTFF1v1 contains a 202 bp insert (consequently missing the first two aminoterminal aa residues of mature mTFF1 ; as described before under "PCR amplification of mouse TFF1 (mTFF1)"). The sequences of the recombinant plasmids are given in figures 1b and 1c. Their complete sequences were compiled from the published sequences of the constituting parts. In addition, relevant sections of the sequences such as PCR fragments and ligation junction points were experimentally verified.

#### ***Protein expression in transformed L. lactis***

*L. lactis* strains were transformed with the plasmids as constructed above. For transformation of the pT1mTFF1 plasmid, *L. lactis* strain MG1363 (Gasson, 1983) was used. For transformation of the pL2mTFF1v1 plasmid, *L. lactis* strain MG1820 (pILPOL) (Maeda and Gasson, 1986) was used.

The expression of the proteins by these transformed *L. lactis* strains was detected by SDS-PAGE.

To prepare culture supernatant fractions, the cells were grown for 20 hours at 28°C in five ml GM17Er medium for the pT1mTFF1 plasmid or GM17Cm medium for the pL2mTFF1v1 plasmid. The cultures were diluted 1/100 in five ml of either GM17Er or GM17Cm medium and grown for 3 hours at 28°C. The cells were collected by centrifugation at 2800 rpm for 20 min and resuspended in five ml of the appropriate medium, i.e., GM9BEr for MG1363 cells or LM9BCm for MG1820 [pILPOL] cells . After

a further five hours of growth the cells were pelleted. The proteins present in the medium fractions were recovered by phenol extraction and ethanol precipitation.

The proteins expressed in the culture supernatant fraction of a *L. lactis* MG1820 control strain compared to *L. lactis* MG1820 strains transformed with  
5 [pILPOL; pL2mTFF1v1] and *L. lactis* MG1363 transformed with [pTREX1; pT1mTFF1]  
are shown in Figure 2. This figure shows an extra protein band of the appropriate size  
(indicated by the arrowhead) in MG1820 [pL2mTFF1v1] and MG1363[pT1mTFF1]  
when compared with the controls. As can be observed from this figure, the expression  
of the recombinant gene is quite low. This renders the observed *in vivo* result  
10 surprising since others use purified trefoil peptides in therapies for the repair of gastric  
and intestinal injury at dramatically higher levels; e.g. Tran *et al.* (1999) used daily  
intrarectal application of human recombinant TTF2 at levels of 2.5 mg/kg body weight  
for five days to obtain a reduction in the inflammatory index of experimentally installed  
colitis in rats (intracolonic administration of dinitrobenzene sulphonic acid in alcohol).

15

#### **Example 2: In vivo testing of MG1363 [pT1mTFF1]**

##### ***Preparation of cells for intragastric administration***

Transformants of *L. lactis* strains, MG1363 [pTREX1], MG1363 [pT1mTFF1]  
20 were streaked on GM17Er plates and grown overnight at 28°C. In each case a single  
colony was subsequently grown overnight at 28°C in 15 ml GM17Er medium. To this  
culture, 15 ml 100% glycerol was added in order to preserve said cells at -20°C. Each  
day, the necessary amount of cells could be inoculated for treatment of mice. To this  
end the culture was diluted 1/200 into 10 ml GM17Er medium. After minimum 20 hours  
25 of growth at 30°C, the cells were collected by centrifugation for 15 min at 2800 rpm.  
The cells were then resuspended in 1 ml M9B without antibiotic.

##### ***In vivo tests in mice with acute colitis***

The effect of the trefoil peptides expressed from these *L. lactis* bacteria was  
30 tested out in mice suffering from acute colitis. Twenty-one female Balb/c mice received  
5% DSS (dextrane sodium sulphate) dissolved in their drinking water during 7 days. In  
this manner, acute colitis was induced (Kojouharoff *et al.*, 1997). For therapeutic  
purposes these mice were orally inoculated daily by means of a gastric catheter using  
100µl bacterial suspension (minimum  $1.10^8$  cells) from day 1 until day 7 of the DSS  
35 treatment. As indicated Six mice were inoculated with MG1363 [pTREX1] cells, six  
mice were inoculated with MG1363 [pT1mTFF1] cells and three mice were not



inoculated (DSS control). On day 8 after the induction of colitis, the mice were sacrificed and examined immunologically and histologically.

Immunological testing of the sera showed that the treated mice did not show an immune response towards the expressed proteins. Serum was taken from the mice which were bled at day 8. This serum was analysed via Western blotting to check whether it contained antibodies against the proteins present in the medium fractions of the *L. lactis* cells. The medium fractions used were derived from the *L. lactis* strains MG1363 [pTREX1] and MG1363 [pT1mTFF1]. An equivalent of 1 ml of concentrated (phenol extraction and ethanol precipitation) medium fractions were analysed by SDS-polyacrylamide (20%) gel electrophoresis. After blotting to nitrocellulose filters, the filters were incubated for 1 hour with the serum solutions of the 4 groups of mice. The serum was diluted 500 times in 20ml nitrocellulose blocking buffer (Blotto: 100ml 10x PBS, 150ml 1M NaCl, 2ml Triton X-100, 25g fat-free milk powder, water up to a total volume of 1 liter). As a secondary antibody, sheep anti-mouse IgG coupled to horseradish peroxidase (HRP) was used. Using the 500 times diluted serum, no signal was detected.

Histological analysis was performed on colons of the treated mice. The colons were cut in the length direction and divided in three equal portions: the distal (nearest to the anus), middle and proximal parts. These colon parts were analysed histologically after an overnight fixation in 3.7% formaldehyde (in PBS), followed by paraffin embedding, ensuring upright positioning of the tissue samples in the paraffin blocks. Of each tissue sample, three parallel 3µm thick longitudinal sections, evenly spaced over the sample, were made. These crosssections were coloured with hematoxylin/eosin. Histological analysis was performed in a blind fashion, meaning that the labels on the slides were covered before scoring the sections. Slides carrying sections obtained from the several groups of mice were randomized before microscopic examination. Each slide was then assigned a histological score (ranging from 0 to 5) according to the symptomatic description as defined in Table 1.

For each mouse and for each colon part, the average score of the three sections was calculated. In the distal and middle parts of the colon, the inflammation consisting of epithelial damage and infiltration were the most pronounced. In the proximal part, almost no inflammation could be observed. The average histological score was calculated for both the distal and the middle colon part per group of animals. The final histological sum score is the sum of the two separate scores (sum score = score of epithelial damage + score of infiltration) and is a measure for the degree of

the inflammation. The histological sum scores of the distal colon part for each of the groups of mice is shown in Figure 3.

From the histological scores for the distal part of the colon as set out in Figure 3, it could be concluded that there is a clear decrease of inflammation upon inoculation of mice with *L. lactis* cells producing trefoil peptides. Mice having received [pT1mTFF1] transformed *L. lactis* cells show a significant reduction of the inflammation of more than 65%.

As can be seen from Figure 3, the inflammatory infiltration and the epithelial damage in the distal part of the colon are significantly decreased following inoculation with recombinant *L. lactis* strains which secrete mTFF1 polypeptide

These results were confirmed in a separate experiment which was conducted equally, including larger groups (group size = 10) and more control groups. Figure 4 shows histological scores (obtained as described above) of healthy control mice (control) and of mice which received DSS as described, either left untreated (DSS) or treated (as described above) with MG1363, MG1363 [pT1TREX1] or MG1363 [pT1mTFF1] as indicated. The experiment shows a clear and significant decrease in the intestinal inflammation in the group of mice treated with MG1363 [pT1mTFF1]

The latter experiment was also evaluated by determining the levels of interleukin-1 $\beta$  (IL-1 $\beta$ ) and interferon- $\gamma$  (IFN- $\gamma$ ), both pro-inflammatory cytokines well known to the skilled. Mice (n=10) were inoculated with the strains indicated as described. Control = healthy mice, DSS = mice receiving 5% DSS in the drinking water without any treatment. The colon was prepared out and areas with equal surface were isolated by means of a punch ( $\varnothing$  = 4 mm). The tissue samples of each group were overlayed with 500  $\mu$ l RPMI + 10% fetal calf serum and incubated overnight at 37°C. The supernatant was collected and titrated for cytokine content by ELISA. The amount of IL-1 $\beta$  and IFN- $\gamma$  in the respective tissues is shown in Figure 5. The results show a clear reduction in these pro-inflammatory cytokines in groups of mice treated with MG1363 [pT1mTFF1]

### **Example 3: Comparison of treatment with MG1363 [pT1TFF1] and purified TFF1**

#### ***Construction of plasmids***

For the expression of mTFF1 from *Pichia pastoris* we constructed the plasmid pPICmTFF1. For this, the mTFF1 gene was PCR amplified as described (PCR amplification of mouse TFF1). This fragment was ligated in the opened *Nae*I restriction site of a derivative of pPIC9 (Invitrogen). The ligation mixture is transformed

to *E. coli* MC1061 and correctly assembled clones were identified by restriction analysis and DNA sequencing (sequence as in Figure 1d). In the resulting plasmid pPICmTFF1, the mTFF1 sequence is fused in frame with the *Sacharomuces cerevisiae*  $\alpha$ -mating factor prepro secretion signal

5

#### **Expression and Purification of mTFF1**

The plasmid pPICmFF1 was transferred to *Pichia pastoris* GST115 by a method as described in Logghe (1995) and positive clones, which had the mTFF1 unit integrated in the his4 locus, were selected by PCR identification. These positive clones  
10 were induced with methanol and screened for expression by protein analysis of culture supernatant and one clone which showed, when compared to the negative control (negative), a particularly high expression of an extra band at 6,5 kDa (GST115::pPICmTFF1) was retained for further work (Figure 6, indicated by arrowhead). The extra protein band was identified as mTFF1 by protein sequencing.  
15 The expression procedure was optimised scaled up and optimised to a 16 l culture and mTFF1 was purified from the culture supernatant.

For this, methanol induced GST115::pPICmTFF1 supernatans was concentrated by tangential filtration ( Millipore proflux M12, cut off 3000 Da) and was dialysed to pH 7.4 in a 0.02 M phosphate buffer. mTFF1 was purified from this concentrate on an ion-  
20 exchange column (Q-column of Biorad). The proteins were eluted form the column by an isocrational salt gradient. The resultant mTFF1 was more than 99% pure and was further concentrated. The final preparation contains less than 160 ng LPS /ml This amount of LPS is within acceptable limits and the pS2 protein can be used in future experiments.

25 Following analysis on a size exclusion column of purified mTFF1 (Superdex 75; Pharmacia) we conclude that 7.5 % of the mTFF1 is in the monomeric form, and 92.5 % is in the dimeric form (Figure 7A). This was confirmed by reducing versus non reducing SDS-PAGE of the purified mTFF1 (Figure 7B).

#### **30 Assessment of biological activity of purified TFF1**

A well know feature of TFF1 protein is that after administration of the protein to Caco-2 cell monolayers it significantly lowers the surface expression of E-cadherine (Liu *et al.*, 1997). We showed a lowering of 10 % of the E-cadherine surface expression after the above described preparation of mTFF1 was administred to Caco-2  
35 monolayers.

***Treatment of murine acute colitis with purified mTFF1:***

For induction of acute colitis mice received 6% dextran sulfate sodium (DSS, MW 40 000) dissolved in drinking water for 7 days (Kojouharoff et al., 1997). Mice used for experiments were age-matched and had received DSS treatment simultaneously. For therapeutic purposes, mice were treated daily with 50 µg mTFF1 in 200 µl PBS before DSS administration from day -7 to 0 (pre-treatment groups), during DSS administration from day 0 to 7 (during-treatment groups) and after DSS administration from day 7 to 14 (post-treatment groups). To study different routes to deliver mTFF1, mice were treated by intraperitoneal (i.p.) injection, intragastric inoculation and rectal administration in each setup. Mice were killed on day 8 after receiving drinking water without DSS for one day (pre-treatment and during-treatment groups) and on day 14 after receiving drinking water without DSS for seven days (post-treatment groups). Non-treated control groups with DSS in drinking water were killed on day 8 and day 14. All groups consisted of 9 mice. Results are represented in Figure 8 and clearly show that in no treatment regime any statistically significant improvement can be observed. This renders the described invention surprising since a clear improvement has been observed (Figure 3 and 4). This means that the delivery of TFF1 through *L. lactis* makes an essential contribution to the observed therapeutic effect.

**Table 1. Symptomatic description of histological scores.**

Score	Epithelium damage	Inflammatory infiltration*
0	Normal morphology	No infiltration
1	Loss of a few goblet cells	Infiltration around the basis of the crypts
2	Widespread loss of goblet cells	Infiltration which reaches the Lamina muscularis mucosae
3	Loss of crypts	Extensive infiltration which reaches the Lamina muscularis mucosae and thickening of the mucosa with prominent oedema
4	Widespread loss of crypts	Infiltration which reaches the Lamina submucosa

\* Inflammatory infiltration includes infiltration of the granulocytes, macrophages and

5 lymphocytes.

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- 20



**CLAIMS**

1. A recombinant micro-organism delivering a trefoil peptide *in vivo*.
- 5 2. A micro-organism according to claim 1, wherein said micro-organism is a bacterial strain.
3. A micro-organism according to claim 2, wherein said micro-organism is a food grade bacterial strain, preferably a gram-positive bacterial strain.
- 10 4. A micro-organism according to claim 3, wherein said bacterial strain is a *Lactococcus* or a *Lactobacillus* species.
- 15 5. A micro-organism according to claim 4, wherein said bacterial strain is *Lactococcus lactis*.
6. A micro-organism according to any of claims 1 to 5 wherein said trefoil peptide is TFF1.
- 20 7. Pharmaceutical composition comprising a micro-organism according to any of claims 1 to 6.
8. Method for the delivery of trefoil peptide to the gastro-intestinal tract comprising the administration of a micro-organism according to any of claims 1 to 6.
- 25 9. Use of a micro-organism according to any of claims 1 to 6 for the manufacture of an agent for the delivery of a trefoil peptide to the gastro-intestinal tract.
- 30 10. Method of treatment of gastric and/or intestinal diseases and/or disorders comprising the administration of a micro-organism according to any of claims 1 to 6.
- 35 11. Method of treatment of lesions caused by gastric and/or intestinal diseases and/or disorders comprising the administration of a micro-organism according to any of claims 1 to 6

12. Use of a micro-organism according to any of claims 1 to 6 for the preparation of a medicament for treatment of gastric and/or -intestinal diseases and/or disorders
- 5
13. Use of a micro-organism according to any of claims 1 to 6 for the preparation of a medicament for treatment of acute gastro-intestinal inflammatory diseases comprising acute colitis, acute flare-ups of Crohn's diseases and ulcerative colitis.
- 10
14. Use of a micro-organism according to any of claims 1 to 6 for the preparation of a medicament for treatment of chronic and spontaneously recurring diseases of the gastro-intestinal tract comprising Crohn's disease (enteritis regionalis) and ulcerative colitis (colitis ulcerosa).
- 15
15. Use of a micro-organism according to any of claims 1 to 6 for the preparation of a medicament for inhibiting the formation of lesions caused by gastric and/or intestinal diseases and disorders.
- 20
16. Method for producing a micro-organism according to any of claims 1 to 6 comprising transforming a micro-organism with a recombinant vector carrying a trefoil peptide coding sequence under the control of a suitable promoter and a suitable secretion signal sequence.
- 25
17. Recombinant vector comprising a trefoil peptide coding sequence under the control of a suitable promoter sequence and a suitable secretion signal sequence.
18. Recombinant vector according to claim 17, having a nucleotide sequence as represented by any of SEQ ID NOs 1, 2, or 3.

1/15

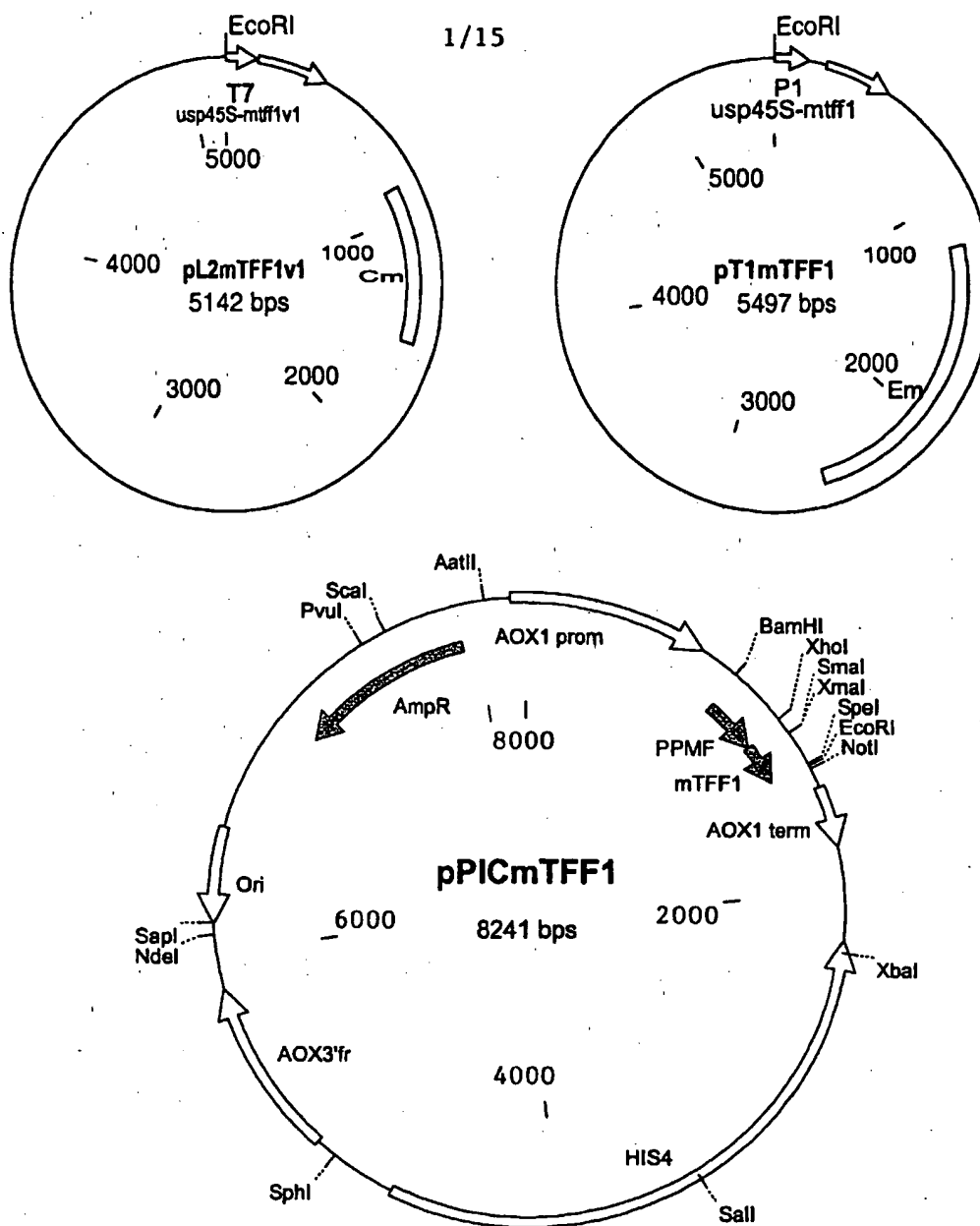


Figure 1a

**Figure 1b:**  
pL2mTFF1v1 (SEQ ID NO 1)

GAATTCGAGCTCGGTACCCGGGGATCTCGATCCCGCGAAATTAATACGACTC  
ACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTGTTTAACTTT  
AAGAAGGAGATATACATATGAAAAAAGATTATCTCAGCTATTTTAATGTC  
TACAGTCATACTTTCTGCTGCAGCCCCGTGTCAGGTGTTTACGCCAGGCC  
CAGGCCCAGGCCCAGGAAGAAACATGTATCATGGCCCCCGGGAGAGGATAA  
ATTGTGGCTTCCCCGGTGTACCGCCAGCAGTGCACGGAGAGAGGTTGCTG  
TTTTGATGACAGTGTCCGGGGATTCCCGTGGTGTCTCCACCCCATGGCCATC  
GAGAACACTCAAGAAGAAGATGTCCCTTCTAACTAGTAGATCCGGCTGCTA  
ACAAAGCCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACT  
AGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAA  
GGAGGAACATATATCCGGATGACCTGCAGGCATGCAAGCTTGGCACTGGCCGT  
CGTTTTTACAACGTGCTGACTGGGAAAACCTGGCGTTACCCAACTTAATCGC  
CTTGCAGCACATCCCCCTTTTCGCCAGCTGATTTTCACTTTTTCATTCTACAA  
ACTGCATAACTCATATGTAAATCGCTCCTTTTTAGGTGGCACAAATGTGAGG  
CATTTTCGCTCTTTCCGGCGAGGCTAGTTACCCCTTAAGTTATTGGTATGACT  
GGTTTTAAGCGCAAAAAAGTTGCTTTTTTCGTACCTATTAATGTATCGTTTTT  
AGAAAACCGACTGTAAAAAGTACAGTCGGCATTATCTCATATTATAAAAGCC  
AGTCATTAGGCCATCTGACAATTCCCTGAATAGAGTTTCATAAACAACTCCTGC  
ATGATAACCATCACAACAGAATGATGTACCTGTAAAGATAGCGGTAAATAT  
ATTGAATTACCTTTATTAATGAATTTTCTGCTGTAAATAGGGTAGAAGGT  
AATTACTATTATTATTGATATTTAAGTTAAACCCAGTAAATGAAGTCCATGG  
AATAATAGAAAGAGAAAAAGCATTTTCAGGTATAGGTGTTTGGGAAACAAT  
TTCCCCGAACCATTATATTTCTCTACATCAGAAAGGTATAAATCATAAAACT  
CTTTGAAGTCATTTCTTACAGGAGTCCAAATACCAGAGAATGTTTTAGATAC  
ACCATCAAAAATTTGTATAAAGTGGCTCTAACTTATCCCAATAACCTAACTCT  
CCGTCGCTATTGTAAACAGTTCTAAAAGCTGTATTTGAGTTTATCACCCCTTG  
TCACTAAGAAAAATAAATGCAGGGTAAAATTTATATCCTTCTTGTTTTATGTT  
TCGGTATAAAACACTAATATCAATTTCTGTGGTTATACTAAAAGTCGTTTGT  
TGGTTCAAATAATGATTAAATATCTCTTTCTCTTCCAATTGTCTAAATCAA  
TTTTATTAAAGTTCATTTGATATGCCTCCTAAATTTTATCTAAAGTGAATT  
TAGGAGGCTTACTTGTCTGCTTTCTTCATTAGAATCAATCCTTTTTTAAAG  
TCAATATTACTGTAAACATAAATATATATTTTAAAAATATCCCACTTTATCCA  
ATTTTAAAGTTTGTGAACATAATGGGTGCTTTAGTTGAAGAATAAAGACCACAT  
TAAAAAGTTGTGCTCTTTTGTGTTTTTTTAAAGGATTTGAGCGTAGCGAAAAA  
TCCTTTTCTTTCTTATCTTGATAATAAGGGTAACTATTGCCGGGATAGACTG  
TAACATTCTCACGCATAAAATCCCCTTTCATTTTCTAATGTAAATCTATTAC  
CTTATTATTAATTCAATTCGCTCATAATTAATCCTTTTTCTTATTACGCAA  
ATGGCCCGATTAAAGCACACCCTTTATCCGTTAATGCGCCATGACAGCCAT  
GATAATTACTAATACTAGGAGAAGTTAATAAATACGTAACCAACATGATTAA  
CAATTATTAGAGGTCATCGTTCAAAATGGTATGCGTTTTTGACACATCCACTA  
TATATCCGTGTCGTTCTGTCCACTCCTGAATCCCATTCAGAAATTCTCTAG  
CGATTCCAGAAGTTTCTCAGAGTCGGAAGTTGACCAGACATTACGAAGTGG  
CACAGATGGTCATAACCTGAAGGAAGATCTGATTGCTTAACTGCTTCAGTTA  
AGACCGAAGCGCTCGTCGTATAACAGATGCGATGATGCAGACCAATCAACAT  
GGCACCTGCCATTGCTACCTGTACAGTCAAGGATGGTAGAAATGTTGTGGT  
CCTTGCACACGAATATTACGCCATTGCTGTCATATTCAAACAGCTCTTCTA  
CGATAAGGGCACAAATCGCATCGTGAACGTTTGGGGTCTTACCGATTTAGC  
AGTTTGATACACTTTCTCTAAGTATCCACCTGAATCATAAATCGGCAAAATA  
GAGAAAAATTGACCATGTGTAAGCGGCCAATCTGATTCCACCTGAGATGCAT  
AATCTAGTAGAATCTCTTCGCTATCAAAATTCACCTCCACCTTCCACTCACC  
GGTTGTCCATTTCATGGCTGAACCTGCTTCTCTGTTGACATGACACACATC  
ATCTCAATATCCGAATAGGGCCCATCAGTCTGACGACCAAGAGAGCCATAAA  
CACCAATAGCCTTAACATCATCCCCATATTTATCCAATATTCGTTCTTAAT  
TTCATGAACAATCTTCATTCTTTCTCTCTAGTCATTATTATTGGTCCATTTC

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Figure 1b - continued -

ACTATTCTCATTCCTTTTCAGATAATTTTAGATTTCGTTTTCTAAATAAGA  
ATATTTGGAGAGCACCGTTCTTATTCAGCTATTAATAACTCGTCTTCCTAAG  
CATCCTTCAATCCTTTTAATAACAATTATAGCATCTAATCTCAACAAACTG  
GCCCCGTTTGTGAACTACTCTTTAATAAAAATAATTTTCCGTTCCCAATTCC  
ACATTGCAATAATAGAAAATCCATCTTCATCGGCTTTTTTCGTCATCATCTGT  
ATGAATCAAATCGCCTTCTTCTGTGTCAAGGTTTAATTTTTTATGTATT  
TCTTTTAACAAACCACCATAGGAGATTAACCTTTTACGGTGTAACCTTCCT  
CCAAATCAGACAAACGTTTCAAATTCCTTTCTTCATCATCGGTCATAAAATC  
CGTATCCTTTACAGGATATTTTGCAGTTTCGTCAATTGCCGATTGTATATCC  
GATTTATATTTTATTTTCGGTATTTTTTATTAAACGTCCTCAAAATCGTTTC  
TGGGACGTTTTAGCGTTTATTTTCGTTTAGTTATCGGCATAATCGTTAAAAACA  
GGCGTTATCGTAGCGTAAAAGCCCTTGAGCGTAGCGTGCTTGCAGCGAAGA  
TGTTGTCTGTTAGATTATGAAAGCCGATGACTGAATGAAATAATAAGCGCAG  
CGTCCTTCTATTTTCGGTTGGAGGAGGCTCAAGGGAGTTTGAGGGAATGAAAT  
TCCCTCATGGGTTTGATTTTAAAAATTGCTTGCAATTTTGCCGAGCGGTAGC  
GCTGGAAAATTTTTGAAAAAATTTGGAATTTGGAAAAAATGGGGGGAAAG  
GAAGCGAATTTTGCTTCCGTACTACGACCCCCCATTAAGTGCCGAGTGCCAA  
TTTTTGTGCCAAAAACGCTCTATCCCACTGGCTCAAGGGTTTGAGGGGTTT  
TTCAATCGCCAACGAATCGCCAACGTTTTCGCCAACGTTTTTTATAAATCTA  
TATTTAAGTAGCTTTATTGTTGTTTTTATGATTACAAAGTGATACACTAATT  
TTATAAAATTATTTGATTGGAGTTTTTTAAATGGTGATTTCAGAATCGAAAA  
AAAGAGTTATGATTTCTCTGACAAAAGAGCAAGATAAAAAATTAACAGATAT  
GGCGAAACAAAAAGGTTTTTCAAATCTGCGGTTGCGGCGTTAGCTATAGAA  
GAATATGCAAGAAAGGAATCAGAACAAAAAATAAGCGAAAGCTCGCGTTT  
TTAGAAGGATACGAGTTTTTCGCTACTTGTTTTTGATAAGGTAATATATCATG  
GCTATTAATAAATACTAAAGCTAGAAATTTTGGATTTTTTATTATATCCTGACT  
CAATTCCTAATGATTGGAAAGAAAAATTAGAGAGTTTGGGCGTATCTATGGC  
TGTCAGTCCTTTTACAGGATATGGACGAAAAAAGATAAAGATACATGGAAT  
AGTAGTGATGTTATACGAAATGGAAAGCACTATAAAAAACCACTATCACG  
TTATATATATTGCACGAAATCCTGTAACAATAGAAAGCGTTAGGAACAAGAT  
TAAGCGAAATTTGGGAATAGTTCAGTTGCTCATGTTGAGATACTTGATTAT  
ATCAAAGGTTTCATATGAATATTTGACTCATGAATCAAAGGACGCTATTGCTA  
AGAATAAACATATATACGACAAAAAAGATATTTTGAACATTAATGATTTTGA  
TATTGACCGCTATATAACACTTGATGAAAGCCAAAAAAGAGAATTGAAGAAT  
TTACTTTTAGATATAGTGGATGACTATAATTTGGTAAATACAAAAGATTTAA  
TGGCTTTTATTCGCCTTAGGGGAGCGGAGTTTGGAAATTTTAAATACGAATGA  
TGTAAGAATATTTGTTTCAACAACTCTAGCGCCTTTAGATTATGGTTTGAG  
GGCAATTATCAGTGTGGATATAGAGCAAGTTATGCAAAGGTTCTTGATGCTG  
AAACGGGGGAAATAAAATGACAAACAAAGAAAAAGAGTTATTTGCTGAAAT  
GAGGAATTAATAAAGAAATTAAGGACTTAAAGAGCGTATTGAAAGATACA  
GAGAAATGGAAGTTGAATTAAGTACAACAATAGATTTATTGAGAGGAGGGAT  
TATTGAATAAATAAAAGCCCCCTGACGAAAGTCGCGACTTCGTTCTTTTTT  
TACCTCTCGGTTATGAGTTAGTTCAAATTCGTTCTTTTTAGGTTCTAAATCG  
TGTTTTCTTGGAATTGTGCTGTTTTATCCTTTACCTTGCTACAAACCCCT  
TAAAAACGTTTTTAAAGGCTTTTAAGCCGCTGTACGTTCCCTTAAG

Figure 1c:  
pT1mTFF1 (SEQ ID NO 2)

GAATTCGATTAAAGTCATCTTACCTCTTTTATTAGTTTTTTCTTATAATCTAA  
TGATAACATTTTTATAATTAATCTATAAACCATATCCCTCTTTGGAATCAAA  
ATTTATTATCTACTCCTTTGTAGATATGTTATAATACAAGTATCAGATCTGG  
GAGACCACAACGGTTTCCCACTAGAAATAATTTTGTTTAACTTTAGAAAGGA  
GATATACGCATGAAAAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTCA  
TACTTTCTGCTGCAGCCCCGTTGTCTAGGTGTTTACGCCCAGGCCAGGCCCA  
GGCCCAGGCCAGGAAGAAACATGTATCATGGCCCCCGGGAGAGGATAAAT  
TGTGGCTTCCCCGGTGTCCCGGATTCCTGCTTCCACCCCATGGCCATCGA  
TGATGACAGTGTCCGGGATTCCCGTGGTGTCTCCACCCCATGGCCATCGA  
GAACACTCAAGAAGAAGAATGTCCCTTCTAACTAGTAGATCCGGCTGCTAAC  
AAAGCCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAG  
CATAACCCCTTGGGGCTCTAAACGGGTCTTGAGGGTTTTTTGTGAAAGG  
AGGAACATATCCGGATGACCTGCAGGCAAGCTCTAGAATCGATACGATTTT  
GAAGTGGCAACAGATAAAAAAAGCAGTTTAAATTTGTGCTGAACTTTTAA  
AACAAGCAAATACAATCATTTGTCGCAACAGATAGCGACAGAGAAGGCGAAAA  
CATTGCCCTGGTCGATCATTCATAAAGCAAATGCCTTTTCTAAAGATAAAACG  
TATAAAGACTATGGATCAATAGTTTAGAAAAAGATGTGATCCGTAGCGGTT  
TTCAAAATTTGCAACCAGGAATGAATTACTATCCCTTTTATCAAGAAGCGCA  
AAAGAAAAACGAAATGATACACCAATCAGTGCAAAAAAGATATAATGGGAG  
ATAAGACGGTTCTGTTCGTGCTGACTTGCACCATATCATAAAATCGAAAC  
AGCAAAGATGGCGGAAACGTAAAAGAAGTTATGGAAATAAGACTTAGAAGC  
AAACTTAAGAGTGTGTTGATAGTGCAGTATCTTAAATTTTGTATAATAGGA  
ATTGAAGTTAAATTAGATGCTAAAAATTTGTAATTAAGAAGGAGTGATTACA  
TGAACAAAAATATAAAATATTTCTCAAACTTTTAAACGAGTGAAAAAGTACT  
CAACCAAATAATAAAACAATTGAATTTAAAAGAAACCGATACCGTTTACGAA  
ATTGGAACAGGTAAAGGGCATTAAACGACGAAACTGGCTAAAATAAGTAAAC  
AGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAA  
ATTAAACTGAATACTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTT  
CAATTCCCTAACAAACAGAGGTATAAAATTTGTTGGGAGTATTCCTTACCATT  
TAAGCACACAAATTATTAATAAAGTGGTTTTTGAAGCCATGCGTCTGACAT  
CTATCTGATTGTGAAGAAGGATTCTACAAGCGTACCTTGGATATTACCGA  
ACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTGCAATTTGCTTAAGC  
TGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAA  
ACTTACCGCCATACCACAGATGTTCCAGATAAATATTGGAAGCTATATACG  
TACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGTTTACTAAAA  
ATCAGTTTCATCAAGCAATGAAACACGCCAAAGTAAACAATTTAAGTACCGT  
TACTTATGAGCAAGTATTGTCTATTTTAAATAGTTATCTATTATTTAACGGG  
AGGAAATAATTCTATGAGTCGCTTTTGTAAATTTGGAAAGTTACACGTTACT  
AAAGGGAATGTAGATAAATTATTAGGTATACTACTGACAGCTTCCAAGGAGC  
TAAAGAGGTCCCTAGCGCTCTTATCATGGGGAAGCTCGGATCATATGCAAGA  
CAAAATAAACTCGCAACAGCACTTGGAGAAATGGGACGAATCGAGAAAACCC  
TCTTTACGCTGGATTACATATCTAATAAGCCGTAAAGGAGACGGGTTCAAAA  
AGGTTTTAAATAAAGGAGAAGCAATCAATGCATTAGCTAGAACTATATTTTTT  
GGACAACGTGGAGAATTTAGAGAACGTGCTCTCCAAGACCAGTTACAAAGAG  
CTAGTGCACTAAACATAATTATTAACGCTATAAGTGTGTGGAACACTGTATA  
TATGGAAAAAGCCGTAGAAGAATTAAGCAAGAGGAGAATTTAGAGAAGAT  
TTAATGCCATATGCGTGGCCGTTAGGATGGGAACATATCAATTTTCTTGGAG  
AATACAAATTTGAAGGATTACATGACACTGGGCAATGAATTTACGTCCTTT  
ACGTATAAAAGAGCCGTTTTTATCTTAATATAACGGCTCTTTTTATAGAAAA  
AATCCTTAGCGTGGTTTTTTTCCGAAATGCTGGCGGTACCCCAAGAATTAGA  
AATGAGTAGATCAAATTATTACGAAATAGAATCAGGAAAAATCAGATCCAACC  
ATAAAAACTAGAACAAATTGCAAGTTAACTAACTCAACGCTAGTAGTGG  
ATTTAATCCCAATGAGCCAACAGAACCAGAGCCAGAAACAGAATCAGAACA  
AGTAACATTGGATTTAGAAATGGAAGAAGAAAAAGCAATGACTTCGTGTGA

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Figure 1c - continued -

ATAATGCACGAAATCGTTGCTTATTTTTTTTTTAAAAGCGGTATACTAGATAT  
AACGAAACAACGAAGTGAATAGAAACGAAAAAGAGCCATGACACATTTATA  
AAATGTTTGACGACATTTTATAAATGCATAGCCCGATAAGATTGCCAAACCA  
ACGCTTATCAGTTAGTCAGATGAACCTCTCCCTCGTAAGAAGTTATTTAATT  
AACTTTGTTTGAAGACGGTATATAACCGTACTATCATTATATAGGGAAATCA  
GAGAGTTTCAAGTATCTAAGCTACTGAATTTAAGAAATGTTAAGCAATCAA  
TCGGAAATCGTTTGATTGCTTTTTTTGTATTCAATTTATAGAAGGTGGAGTTT  
GTATGAATCATGATGAATGTAAACTTATATAAAAAATAGTTTATTGGAGAT  
AAGAAAATTAGCAAATATCTATACACTAGAAACGTTTAAAGAAAGAGTTAGAA  
AAGAGAAAATATCTACTTAGAAAACAAAATCAGATAAGTATTTTTCTTCGGAGG  
GGGAAGATTATATATATAAGTTAATAGAAAATAACAAAATAATTTATTTCGAT  
TAGTGGAAAAAAATTGACTTATAAAGGAAAAAAATCTTTTTCAAAACATGCA  
ATATTGAAACAGTTGAATGAAAAAGCAAACCAAGTTAATTAACAACCTATT  
TTATAGGATTTATAGGAAAGGAGAACAGCTGAATGAATATCCCTTTTGTTGT  
AGAACTGTGCTTCATGACGGCTTGTTAAAGTACAAATTTAAAAATAGTAAA  
ATTCGCTCAATCACTACCAAGCCAGGTAAAAGCAAAGGGGCTATTTTTGCGT  
ATCGCTCAAAATCAAGCATGATTGGCGGTCGTGGTGTGTTCTGACTTCCGA  
GGAAGCGATTCAAGAAAATCAAGATACATTTACACATTGGACACCCAACGTT  
TATCGTTATGGAACGTATGCAGACGAAAACCGTTCATACACGAAAGGACATT  
CTGAAAACAATTTAAGACAAATCAATACCTCTTTATTGATTTTGATATTCA  
CACGGCAAAAGAACTATTTTCAGCAAGCGATATTTTAAACACCGCTATTGAT  
TTAGGTTTTATGCCTACTATGATTATCAAATCTGATAAAGGTTATCAAGCAT  
ATTTTGTTTTAGAAACGCCAGTCTATGTGACTTCAAAATCAGAATTTAAATC  
TGTCAAAGCAGCCAAAATAATTTCCGCAAAATATCCGAGAATATTTTGAAAG  
TCTTTGCCAGTTGATCTAACGTGTAATCATTTTGGTATTGCTCGCATACCAA  
GAACGGACAATGTAGAATTTTTTGATCCTAATTACCGTTATTCTTTCAAAGA  
ATGGCAAGATTGGTCTTTTCAAACAAAACAGATAATAAGGGCTTTACTCGTTCA  
AGTCTAACGGTTTTAAGCGGTACAGAAGGCAAAAACAAGTAGATGAACCCT  
GGTTTAATCTCTTATTGCACGAAACGAAATTTTCAGGAGAAAAGGGTTTAAT  
AGGGCGTAATAACGTGATGTTTACCCTCTCTTTAGCCTACTTTAGTTCAGGC  
TATTTCAATCGAAACGTGCGAATATAATATGTTTGAGTTTAATAATCGATTAG  
ATCAACCCTTAGAAGAAAAAGTAATCAAATTTGTTAGAAGTGCCATTTC  
AGAAAATATCAAGGGGCTAATAGGGAATACATTACCATTCTTTGCAAAGCT  
TGGGATTCAAGTGATTTAACCAAGTAAAGATTTATTTGTCCGTCAAGGGTGGT  
TTAAATTTCAAGAAAAAAGAAGCGAACGTCAACGTGTTCAATTTGTCAGAATG  
GAAAGAAGATTTAATGGCTTATATTAGCGAAAAAAGCGATGTATACAAGCCT  
TATTTAGTGACGACCAAAAAAGAGATTAGAGAAGTGCTAGGCATTCTGAAC  
GGACATTAGATAAATTGCTGAAGGTACTGAAGGCGAATCAGGAAATTTTCTT  
TAAGATTAAACCAGGAAGAAATGGTGGCATTCAACTTGCTAGTGTTAAATCA  
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AAACAAGCTAGCAGAACGCCCTAAAACGGACACACAACCTCGATTTGTTTAGC  
TATGATACAGGCTGAAAAATAAAACCCGCACTATGCCATTACATTTATATCTA  
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ACAGAGTAAGATTTAATTAATTATTAGGGGAGAAGGAGAGAGTAGCCCGA  
AACTTTTTAGTTGGCTTGGACTGAACGAAGTGAGGGAAGGCTACTAAAACG  
TCGAGGGGCGAGAGCGAAGCGAACACTTGATTTTTTAATTTCTATCTT  
TTATAGGTCATTAGAGTATACTTATTTGTCCTATAAACTATTTAGCAGCATA  
ATAGATTTATTGAATAGGTCATTTAAGTTGAGCATATTAGAGGAGGAAAATC  
TTGGAGAAATATTTGAAGAACCCGATTACATGGATTGGATTAGTTCCTGTGG  
TTACGTGGTTTTTAATAAAAGTAGTGAATTTTGATTTTGGTGTGTGTGT  
CTTGTGTTAGTATTTGCTAGTCAAAGTGATTAAATA

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Figure 1d:  
pPICmTFF1 (SEQ ID NO 3)

AGATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTGGCCATCCGACATCCACAGGTCCAT  
TCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAAACGAGG  
ACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGGCCATCGAAAAACAGCCCAGTTATTGGGCTT  
GATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGCTACTAACACCATGACTTTATTAGCCTGTC  
TATCCTGGCCCCCTGGCGAGGTTTCATGTTTGTATTTCCGAATGCAACAAGCTCCGCATTACAC  
CCGAACATCACTCCAGATGAGGGCTTCTGAGTGTGGGGTCAAATAGTTTTCATGTTCCCCAAATGG  
CCCCAACTGCAGTTTAAACGCTGTCTTGGAACTAATATGACAAAAGCGTGATCTCATCCAAGA  
TGAACATAAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAACTTCCAAAAGTCGC  
CATACCGTTTGTCTTGTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAAATGCTTAGCG  
CAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATGGGGAAACACCCGCT  
TTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAATACTGCTGAT  
AGCCTAACGTTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATATATAAACAGAA  
GGAAGCTGCCCTGTCTTAAACCTTTTATCATCATTATTAGCTTACTTTCATAATTGCGACT  
GGTTCCAATTGACAAGCTTTTGATTTTAAACGACTTTTAAACGACAACTTGAGAAGATCAAAAACAA  
CTAATTATTCGAAGGATCCAAACGATGAGATTTCTTCAATTTTACTGCAGTTTATTTCGCAGCA  
TCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATCCGGCTGAA  
CTGTGTCATCGGTTACTCAGATTTAGAAGGGGATTTTCGATGTTGCTGTTTGGCATTTTCCAACAGC  
ACAAATAACGGGTATTGTTTATAAACTACTATTGCGCAGCATTGCTGCTAAAGAAGAAGGGGTA  
TCTCTCGAGAAAAGAGAGGCTGAAGCCCAGGCCAGGCCAGGCCAGGCCAGGGAAGAAACATGT  
ATCATGGCCCCCGGAGAGGATAAATTGTGGCTTCCCCGGTGTACCGCCAGCAGTGCACGGAG  
AGAGGTTGCTGTTTGTATGACAGTGTCCGGGATTCCCGTGGTGTCTCCACCCATGGCCATCGAG  
AACACTCAAGAAGAAGATGTCCCTTCTAACTAGTGGCGTAGAATTCCCTAGGGCGGCCGCGAATT  
AATTCGCCCTTAGACATGACTGTTCTCAGTTCAAGTTGGGCACTTACGAGAAGACCGGTCTTGCTA  
GATTCTAATCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGGCTTCATTTTGTACTT  
TTTTATTGTAACTATATAGTATAGGATTTTTTTTGTCAATTTGTTTCTTCTCGTACGAGCTTGC  
TCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTGGGAAATCATTCGAGT  
TTGATGTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACAGAAGATTAAGTGAGAAGTTTCGTTT  
GTGCAAGCTTATCGATAAGCTTTAATGCGGTAGTTTATCACAGTTAAATTGCTAACGCAGTCAGGC  
ACCGTGTATGAAATCTAACAATGCGCTCATCGTCATCTCGGCACCGTCACCTGGATGCTGTAGG  
CATAGGCTTGGTTATGCCGGTACTGCCGGGCTCTTGCGGGATATCGTCCATTCCGACAGCATCGC  
CAGTCACTATGGCGTCTGCTAGCGCTATATGCGTTGATGCAATTTCTATGCGCACCCGTTCTCGG  
AGCACTGTCCGACCGCTTTGGCCGCCGCCAGTCCTGCTCGCTTCGCTACTTGGAGCCACTATCGA  
CTACGCGATCATGGCGACCACACCCGTCCTGTGGATCTATCGAATCTAAATGTAAGTTAAATCTC  
TAAATAAGTTAAATAGTCCCAGTTTCTCATACGAACTTAAACAGCATTGCGGTGAGCATCTAGAC  
CTTCAACAGGACGCCAGATCCATCTGCTTGGCCAATATGTTTCAGTCCCTCAGGAGTTACGCTCTT  
GTGAAGTGATGAACCTCTGGAAGGTTGCAGTGTAACTCCGCTGTATTGACGGGCATATCCGTACG  
TTGGCAAAGTGTGGTTGGTACCGGAGGAGTAATCTCCACAACCTCTCTGGAGAGTAGGCACCAACAA  
ACACAGATCCAGCGTGTGTACTTGATCAACATAAGAAGAAGCATTCTCGATTTCAGGATCAAGT  
GTTTCAGGAGCGTACTGATTGGACATTTCCAAAGCCTGCTCGTAGGTTGCAACCGATAGGGTTGTAG  
AGTGTGCAATACACTTGGCTACAATTTCAACCCCTTGGCAACTGCACAGCTTGGTTGTGAACAGCAT  
CTTCAATTCTGGCAAGCTCCTTGTCTGTATATCGACAGCCAACAGAATCACCTGGGAATCAATA  
CATGTTTCAGCTTGAGACAGAAGGCTGAGGCAACGAAATCTGGATCAGCGTATTTATCAGCAATAA  
CTAGAACTTCAGAAGGCCAGCAGGCTGTCAATCTACACAGGCTGATGTGTCAATTTTGAACCA  
TCATCTTGGCAGCAGTAACGAAGTGGTTTCTTGGACCAATATTTTGTCACTTAGGAACAGTTT  
CTGTTCCGTAAGCCATAGCAGCTACTGCCCTGGGCGCCTCTGCTAGCAGCATACACTTAGCACCAA  
CCTGTGGGCAACGTAGATGACTTCTGGGGTAAGGGTACCATCCTTCTTAGGTGGAGATGCAAAAA  
CAATTTCTTTGCAACCAGCAACTTTGGCAGGAACACCCAGCATCAGGGAAGTGAAGGCAGAATTG  
CGGTTCCACCAGGAATATAGAGGCCAATTTCTCAATAGGTCTTGCAAAACGAGAGCAGACTACAC  
CAGGGCAAGTCTCAACTTGCAACGCTCCGTTAGTTGAGCTTCATGGAATTTCTTGACGTTATCTA  
TAGAGAGATCAATGGCTCTCTTAACGTTATCTGGCAATTGCATAAGTTCCTCTGGGAAGGAGCTT  
CTAACACAGGTGTCTTCAAAGCGACTCCATCAAACCTTGGCAGTTAGTTCTAAAGGGCTTTGTCTAC  
CATTTTGACGAACATTGTGCAATTTGGTTGACTAATTCCATAATCTGTTCCGTTTTCTGGATAG  
GACGACGAAGGGCATCTCAATTTCTGTGAGGAGGCCTTAGAAACGTCAATTTTGCACAATTCAA  
TACGACCTTCAGAAGGGACTTCTTTAGGTTTGATTCTTCTTTAGGTTGTTCTTGGTGTATCCTG



Figure 1d - continued -

GCTTGGCATCTCCTTTCTCTAGTGACCTTTAGGGACTTCATATCCAGGTTTCTCTCCACCTCGT  
CCAACGTACACCGTACTTGGCACATCTAATAATGCAAAATAAAATAAGTCAGCACATTTCCAGG  
CTATATCTTCTTGGATTTAGCTTCTGCAAGTTCATCAGCTTCCCTCCCTAATTTTAGCGTTCAACA  
AACTTCGTCGTCAAATAACCGTTTGGTATAAGAACCCTTCTGGAGCATTGCTCTTACGATCCCACA  
AGGTGGCTTCCATGGCTCTAAGACCCTTTGATTGGCCAAAACAGGAAGTCCGTTCCAAGTGACAGA  
AACCAACACCTGTTTGTTCACCAAAATTTCAAGCAGTCTCCATCACAATCCAATTTCGATACCCA  
GCAACTTTTGAGTTGCTCCAGATGTAGCACCTTTATACCACAAACCGTGACGACGAGATTGGTAGA  
CTCCAGTTTGTGTCTTATAGCCTCCGGAATAGACTTTTTGGACGAGTACACCAGGCCCAACGAGT  
AATTAGAAGAGTCAGCCACCAAGTAGTGAATAGACCATCGGGGCGGTACAGTCAAGACGCCA  
ACAAAATTTCACTGACAGGGAACTTTTGACATCTTCAGAAAGTTCGTATTCAGTAGTCAATTGCC  
GAGCATCAATAATGGGGATTATACCAGAAGCAACAGTGGAAAGTCACATCTACCAACTTTGCGGTCT  
CAGAAAAAGCATAAACAGTTCTACTACCGCCATTAGTGAAACTTTTCAAATCGCCCAGTGGAGAAG  
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TAACCTTAGCGCTGGGATCATCCTTTGGACAACCTTTCTGCCAAATCTAGGTCCAAAATCACTT  
CATTGATACCATTATTGTACAACCTTGAGCAAGTTGTGCGATCAGCTCCTCAAATTTGGTCTCTGTAA  
CGGATGACTCAACTTGCACATTAACCTGAAGCTCAGTCGATTGAGTGAACCTGATCAGGTTGTGCA  
GCTGGTCAGCAGCATAGGGAACACCGCTTTTCTACCAAACCTCAAGGAATTATCAAACCTCTGCA  
CACTTGCGTATGCAGGTAGCAAGGGAATGTCACTTGAAGTCGGACAGTGAAGTGTAGTCTTTGAG  
AAATTTCTGAAGCCGTATTTTATTATCAGTGAGTCAGTCATCAGGAGATCCTCTACGCCGGACGCA  
TCGTGGCCGACCTGCAGGTCGGCATCACCGCGCCACAGGTGCGGTGTGCTGGCGCTTATATCGCCG  
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TGGTGGCAGGCCCCGTGGCCGGGGACTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGGCG  
CGGCGGTGCTCAACGGCCTCAACCTACTACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAG  
AGCGTCGAGTATCTATGATTGGAAGTATGGGAATGGTGATACCCGCATTCTTCAGTGTCTTGAGGT  
CTCCTATCAGATTATGCCAACTAAAGCAACCGGAGGAGGAGATTTATGGTAAATTTCTCTGACT  
TTTGGTCATCAGTAGACTCGAACTGTGAGACTATCTCGGTTATGACAGCAGAAATGTCTCTTTGG  
AGACGTAATGAAGTCCCACCAATAAAGAAATCCTTGTATCAGGAACAAACTTCTTGTTCGAA  
CTTTTTCGGTGCTTGAACATAAAATGTAGAGTGGATATGTGCGGTAGGAATGGAGCGGGCAAAT  
GCTTACCTTCTGGACCTTCAAGAGGTATGTAGGGTTTGTAGATACTGATGCCAACTTCAGTGACAA  
CGTTGCTATTTCTGTTCAAACCATTCGAATCCAGAGAAATCAAAGTTGTTTGTCTACTATTGATCC  
AAGCCAGTGGCGTCTTGAACTGACAATAGTGTGCTCGTGTTCGAGGTATCTTTGTATGAATAA  
ATCTAGTCTTTGATCTAAATAATCTTGACGAGCCAAGGCGATAAATACCCAAATCTAAAACTCTTT  
TAAACGTTAAAGGACAAGTATGTCTGCCTGTATTAACCCCCAAATCAGCTCGTAGTCTGATCCT  
CATCAACTTTAGGGGGCACTATCTTGTTTTAGAGAAATTTGCGGAGATGCGATATCGAGAAAAAGGT  
ACGCTGATTTTAAACGTGAAATTTATCTCAAGATCTCTGCTCGCGCTTTTCGGTGAACGGTGA  
AAACCTCTGCACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAG  
ACAAGCCCGTCAGGGCGCTCAGCGGTGTGGCGGTGTGCGGGCGCAGCCATGACCCAGTCACG  
TAGCGATAGCGGAGTGATACTGGCTTAACATATGCGGCATCAGAGCAGATTGTACTGAGAGTGCAC  
CATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCTCTTCCGCT  
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GCGGTAATACGGTTATCCACAGAAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAG  
CAAAAGGCCAGGAACCGTAAAAAGGCCGCTTGTGCGGTTTTTCCATAGGCTCCGCCCCCTGAC  
GAGCATCACAAAAATCGACGCTCAAGTCAGAGTGGCGAAACCCGACAGGACTATAAAGATACAG  
GCGTTTTCCCCCTGGAAGCTCCCTCGCTCTCCTGTTCCGACCTGCGGCTTACCGGATACCTG  
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TTATCCGGTAACTATCGTCTTGTAGTCCAACCCGTAAGACACGACTTATCGCCACTGCGACGAGCC  
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GACGTCAGATGGAAACGAAACTACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTC  
ACCTAGATCCTTTTAAATTAATAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGG  
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ATAGTTGCCTGACTCCCCGTGCTGTAGATAACTACGATACGGGAGGGCTTACCATCTGCCCCAGT

**Figure 1d – continued -**

GCTGCAATGATACCGCGAGACCCACGCTCACC GGCTCCAGATTTATCAGCAATAAACAGCCAGCC  
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AGTAAGTTGGCCGAGTGTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATG  
CCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATG  
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TCCAGTTCGATGTAACCCACTCGTGCAACCAACTGATCTTCAGCATCTTTTACTTTTACCAGCGTT  
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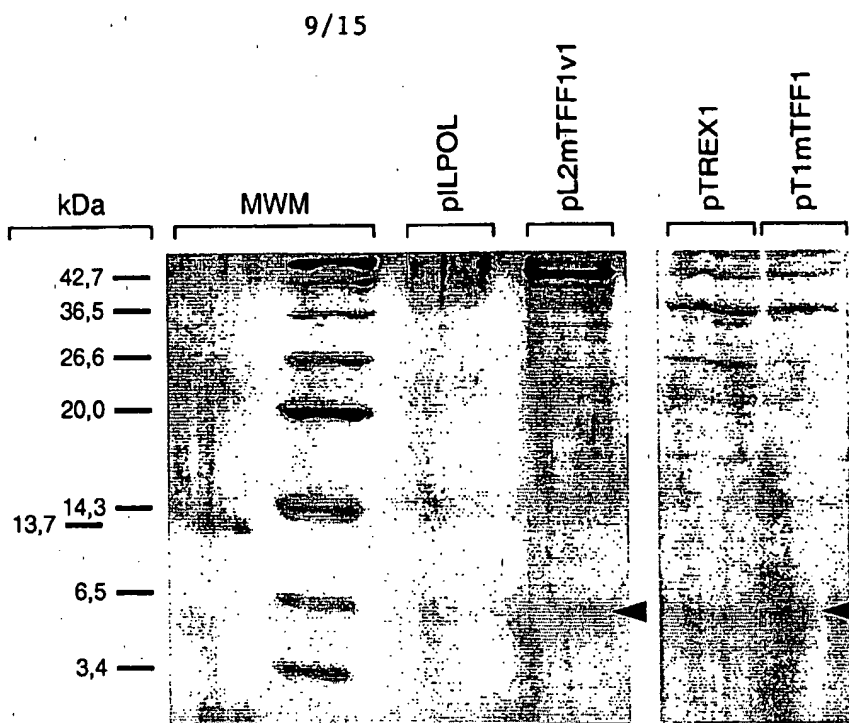


Figure 2

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Distal Colon

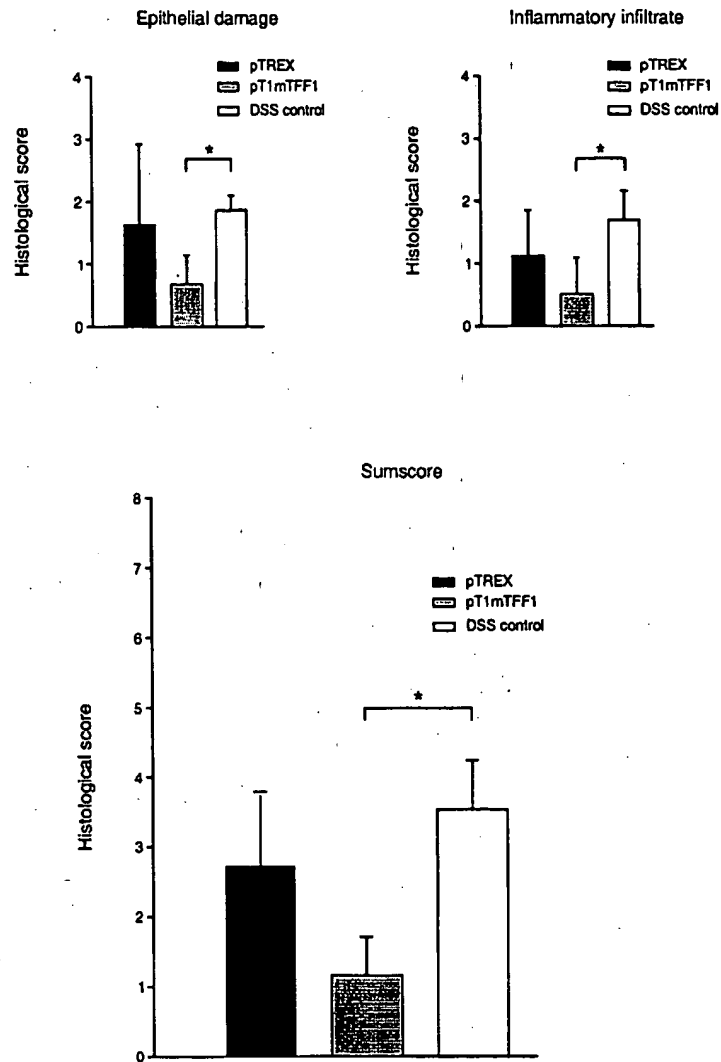


Figure 3

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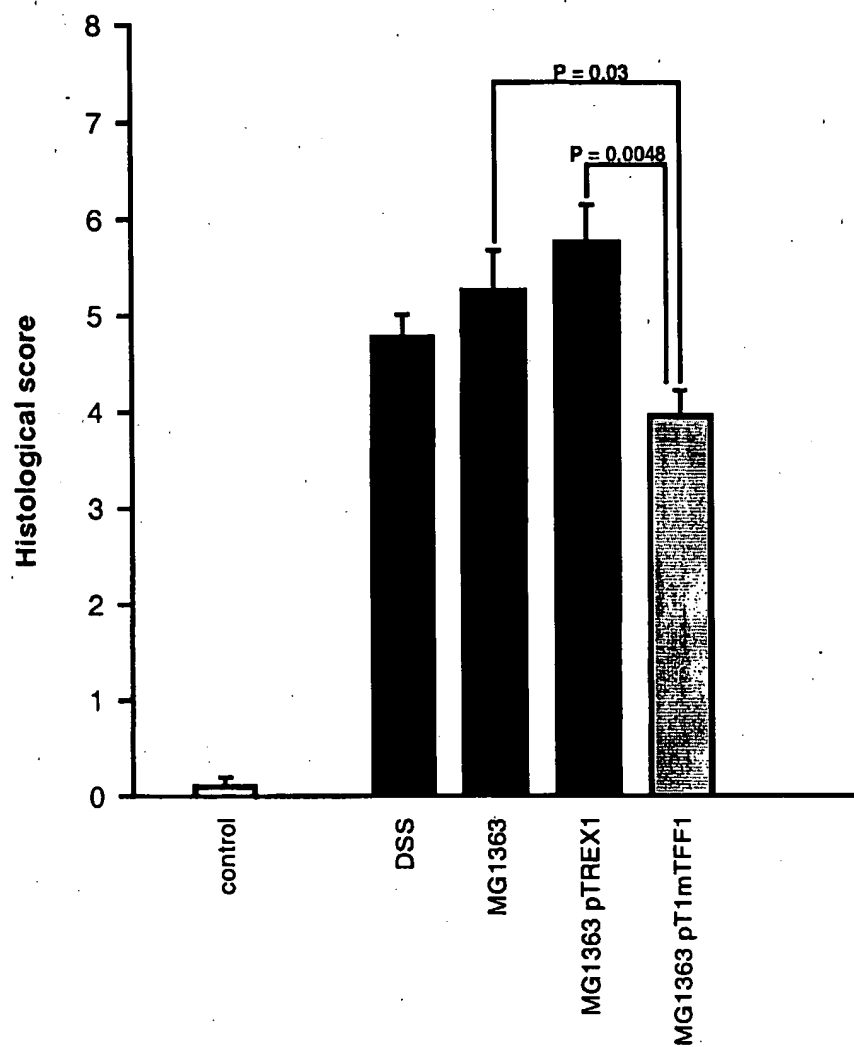


Figure 4

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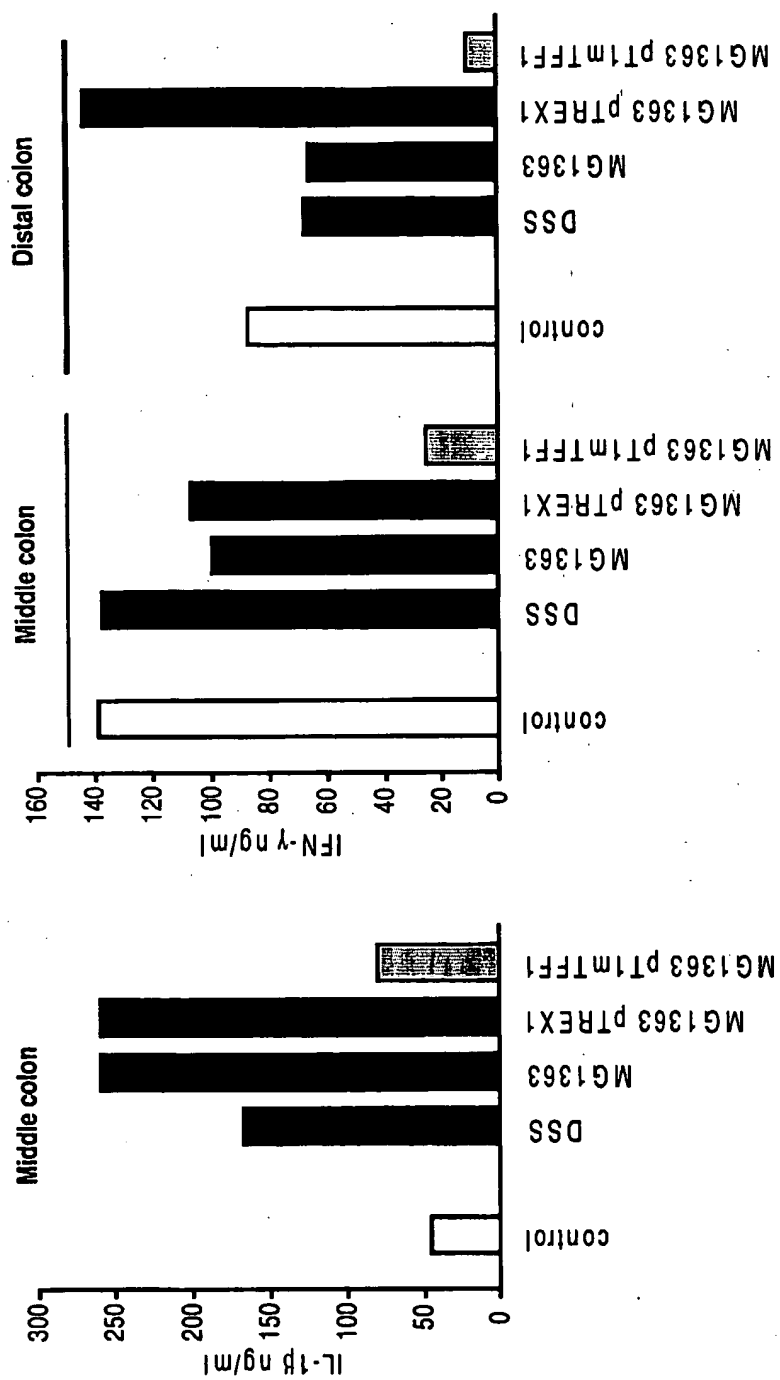


Figure 5

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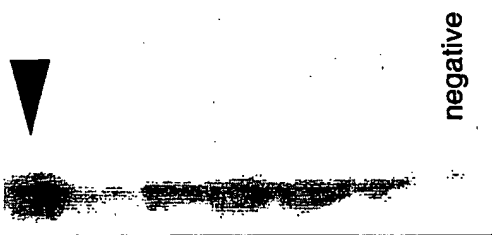
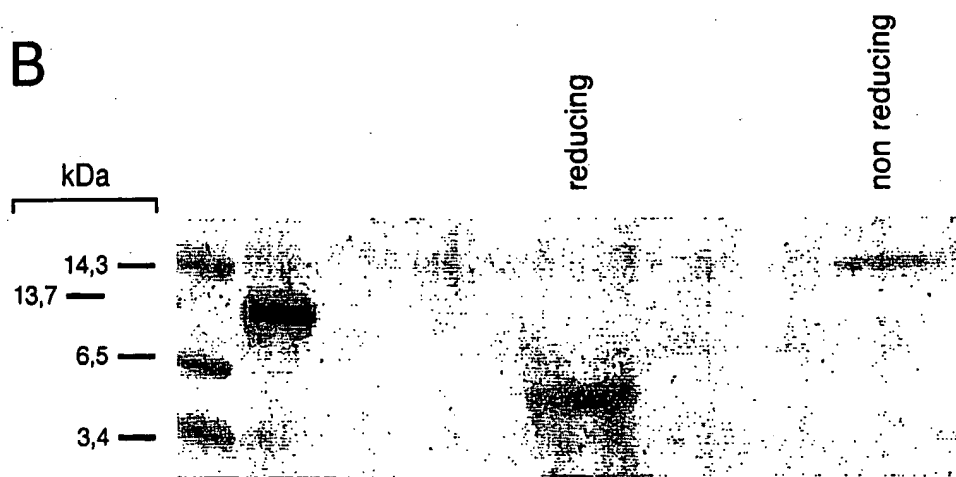
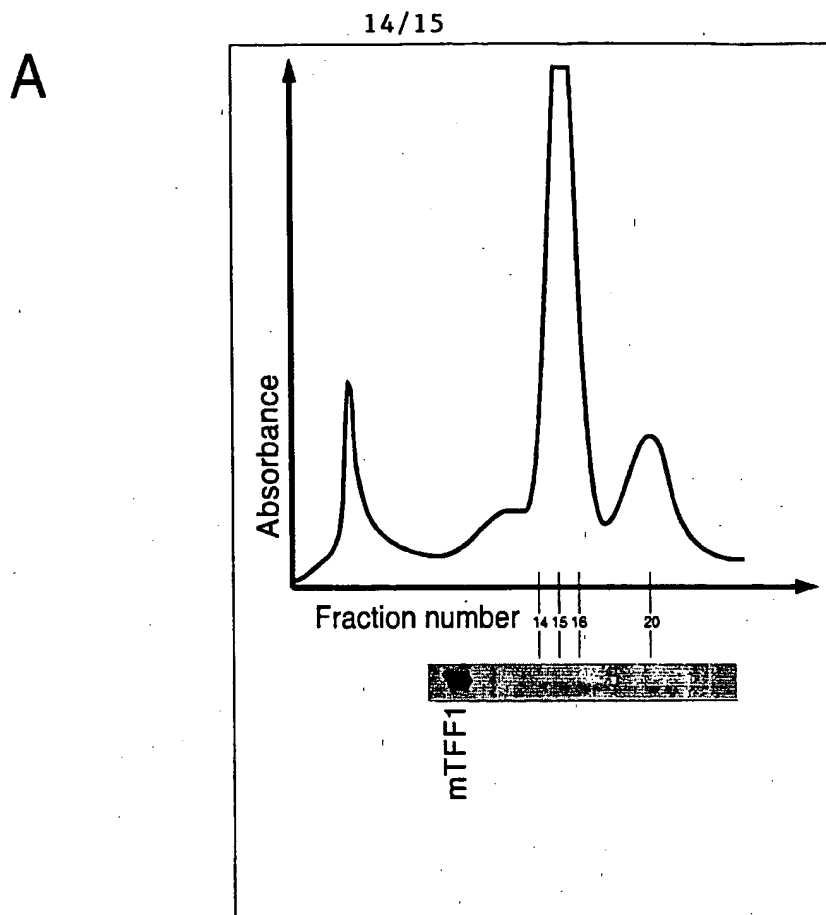


Figure 6



**Figure 7**



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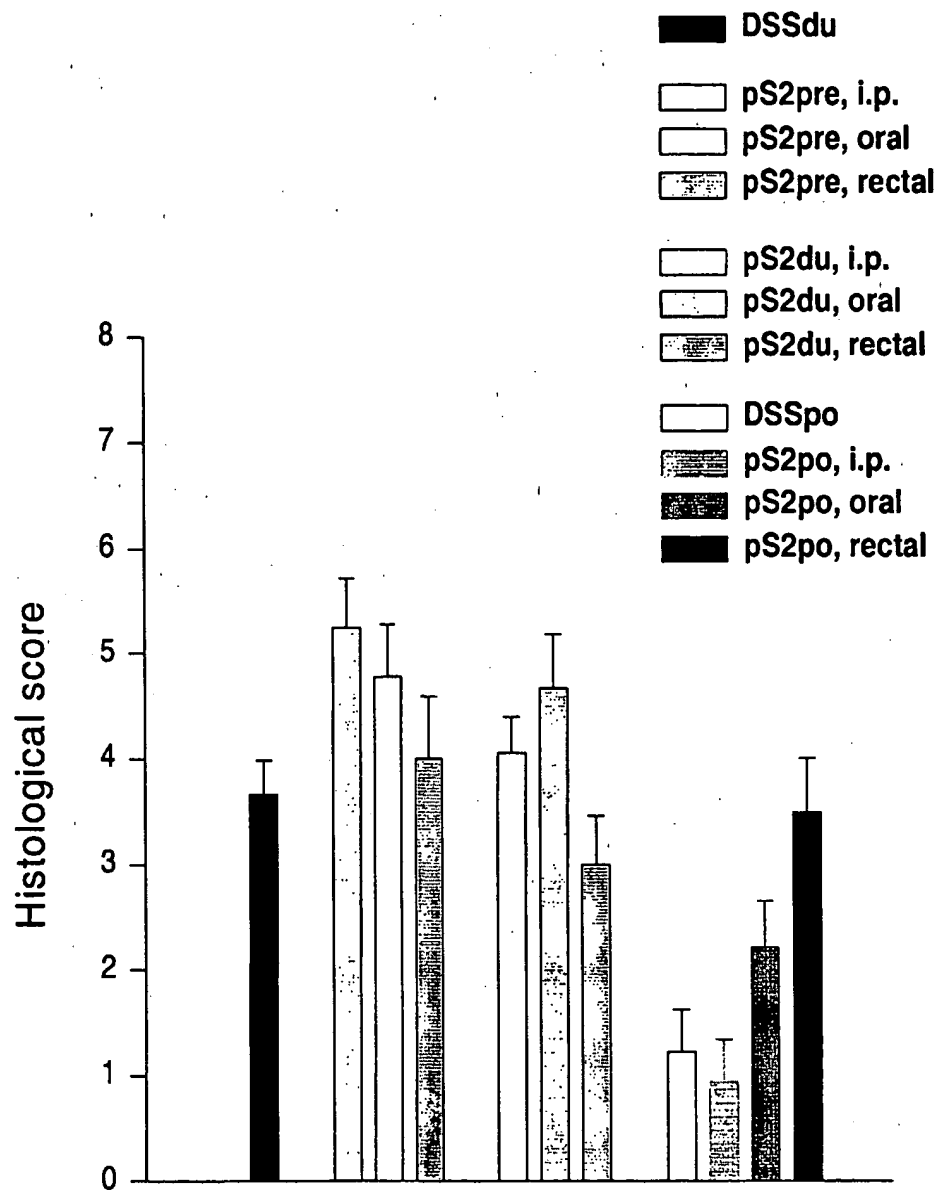


Figure 8

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